INVESTIGATION OF BARCELONA AND DU CHILLY FILBERT NUTS

II. ISOLATION, NUTRITIONAL EVALUATION AND AMINO ACID DISTRIBUTION OF FILBERT GLOBULINS [1]

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In addition to oil and carbohydrate, the filbert nut contains about 20% protein. Domestic filbert oils have been investigated in this laboratory and the findings reported elsewhere (Fang and Bullis, '49).

The nutritive properties of European filbert nuts have been studied by Cajori ('20), DeCaro and Franceschini ('39), Bevilotti ('42) and Engels ('43). No information is available in the literature on the nutritive properties of domestic filbert nut protein. The present work is concerned with the nutritional value of globulin prepared from Barcelona and Du Chilly varieties of filbert nuts (*Corylus Avellana*), which are grown commercially in Oregon and Washington.

EXPERIMENTAL

Isolation of globulin

After removing the oil by extraction with petroleum ether, the nuts were ground into a fine powder by means of a Wiley mill. The oil-free meal was then extracted with a 10% sodium

[1] The data in this paper are taken from a thesis presented by Sheng Chung Fang to the Faculty of the Graduate School of Oregon State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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chloride solution at room temperature overnight. The mass was drained through cheese cloth and the filtrate centrifuged to remove the particles which passed through the cloth. The supernatant liquid was placed in a cellophane bag and dialyzed until free from chloride ions. The precipitated globulin was removed by centrifugation, washed several times with methyl alcohol and finally with ether, and allowed to dry at room temperature. The globulins prepared from both Barcelona and Du Chilly filbert nuts were slightly yellow in color and granular in appearance. The ash, nitrogen, carbon and hydrogen were determined and the results are summarized in table 1.

TABLE 1

Elementary composition of globulins from Barcelona and Du Chilly filbert nuts

GLOBULIN PREPARATION	C	H	N	ASH	AMOUNT ISOLATED FROM OIL-FREE MEAL
	%	%	%	%	%
Barcelona filbert globulin	49.71	6.87	16.50	0.73	18.1
Du Chilly filbert globulin	49.76	6.90	16.36	0.47	22.8

Amino acid analysis

The globulin fractions from both Barcelona and Du Chilly filbert nuts were assayed by a slight modification of the microbiological procedure of Henderson and Snell ('48). As a reference, a sample of vitamin-free casein was analyzed. The results are given in table 2, where they are compared with the range of values in the literature found by microbiological assay of this protein. The values obtained for 16 amino acids found in casein were in very good agreement with those in the literature, except that slightly higher values were found in the cases of proline, tyrosine, and threonine and lower in the case of aspartic acid. The globulins, when compared to casein, were high in arginine and cystine and low in lysine, proline and methionine. The amino acids were rather well balanced for a plant protein.

Growth response of rats to rations using filbert globulins as the sole source of protein, with or without supplementation

Animal feeding experiments were then carried out, since it was desirable to determine the availability of the amino acids as well as to confirm the microbiological results.

Test rations were made up according to the method of Bubl and Butts ('48) except that the protein level was re-

TABLE 2

Amino acid composition of filbert nut globulins and casein

AMINO ACID [1]	ASSAY ORGANISM [2]	CASEIN [3]		BARCELONA FILBERT GLOBULIN	DU CHILLY FILBERT GLOBULIN
		Found	Literature [4,5]		
		%	%	%	%
Arginine	S	3.39	3.78 ± 0.65 [6]	14.44	14.50
Aspartic acid	M	5.70	7.40 ± 0.03	4.40	4.22
Cystine	A	0.70	0.36 ± 0.04	2.22	2.47
Glutamic acid	A	21.3	21.70 ± 1.7	19.0	19.4
Histidine	S	3.00	3.03 ± 0.24	2.41	1.96
Isoleucine	A	8.30	7.60 ± 0.35	5.13	4.97
Leucine	A	10.68	10.25 ± 0.30	10.0	6.14
Lysine	M	7.96	8.07 ± 0.30	2.07	1.99
Methionine	S	2.61	2.69 ± 0.26	0.81	0.84
Phenylalanine	C	5.27	5.45 ± 0.34	3.84	3.54
Proline	M	15.30	11.6 ± 0.7	4.40	4.22
Serine	M	6.47	7.5	5.39	5.59
Threonine	A	6.23	4.28 ± 0.28	5.31	5.59
Tryptophan	A	1.36	1.28	1.45	1.52
Tyrosine	S	7.22	5.8 ± 0.7	3.26	3.23
Valine	C	7.06	7.15 ± 0.4	3.74	3.38

[1] The amino acids used as standards were dried *in vacuo* at 45°C. and kept in a desiccator containing anhydrous calcium chloride. The DL-forms of tryptophan, threonine, aspartic acid, phenylalanine, serine, alanine, methionine, leucine, isoleucine and valine were employed; the natural isomers of the other acids were used.

[2] S = *Streptococcus faecalis* R.
A = *Lactobacillus arabinosus.*
C = *Lactobacillus casei.*
M = *Leuconostoc mesenteroides* P-60.

[3] Results were calculated to 16.0 gm of nitrogen.

[4] Block ('45).

[5] Henderson and Snell ('48).

[6] Range of values as reported in literature.

duced to 16.5%. In the unsupplemented diets, 16.5% of either Barcelona globulin, Du Chilly globulin or casein was used. In the amino acid supplemented diets, 14.3% of protein plus 2.0% of DL-lysine and 1.2% of DL-methionine were fed. The animals were young rats 28 to 30 days of age and of the Long-Evans strain. They were placed in individual wire cages and subjected to paired feeding, using casein as the reference protein.

In all experiments the rats receiving the unsupplemented Barcelona or Du Chilly globulin diet either died or failed to gain weight, while those receiving the casein diet in like amounts made fairly satisfactory growth.

After three weeks of paired feeding with the amino acid supplemented diet it was found that the growth response to the filbert globulin rations was equal to that of the casein ration. It is undoubtedly true that filbert globulins are deficient in these two essential amino acids (lysine and methionine).

CONCLUSION

The protein of the filbert nut can be quite easily isolated by extraction with a 10% sodium chloride solution. On the basis of amino acid values, the globulin fractions of both Barcelona and Du Chilly varieties are relatively low in lysine and methionine.

Feeding experiments with rats confirmed the microbiological results, which indicated that filbert globulin alone is not adequate as a source of protein. However, after supplementation with lysine and methionine this protein promoted the growth of rats as well as did casein.

SUMMARY

Sixteen amino acids were determined by microbiological assay in globulins isolated from filbert nuts. A sample of vitamin-free casein was analyzed and the results compared with values reported in the literature in order to judge the accuracy of these determinations.

The microbiological results were confirmed by growth experiments; namely, that these globulins are critically low in lysine and methionine.

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STUDIES IN AMINO ACID UTILIZATION

IV. THE MINIMUM REQUIREMENTS OF THE INDISPENSABLE AMINO ACIDS FOR MAINTENANCE OF THE ADULT WELL-NOURISHED MALE ALBINO RAT [1]

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ONE FIGURE

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INTRODUCTION

Inherent in every feeding situation, whether it be that of engendering growth, rehabilitation or the maintenance of health in an adult animal, is the problem of supplying the amino acid needs of the "continuing metabolism." In a previous communciation from this laboratory (Wissler et al., '48) it was demonstrated that 9 amino acids were indispensable for the maintenance of appetite, weight and nitrogen equilibrium in the adult rat. Moreover, with a diet containing only these 9 amino acids as the source of amino nitrogen, adult animals could be maintained in nitrogen equilibrium. The amino acids required were tryptophan, lysine, histidine, phenylalanine, threonine, methionine, valine, leucine and isoleucine. These are 9 of the 10 amino acids which have been

[1] The research which this paper reports was undertaken in cooperation with the Navy Department Office of Naval Research and the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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found to be indispensable for the optimum growth of the young rat (Rose, '38) and the same 9 which were found to be necessary for the rehabilitation of the depleted rat (Frazier et al., '47). Having established the qualitative amino acid requirements of the rat for maintenance, it became of further interest to determine the minimum quantity of each of these essential substances necessary to maintain nitrogen equilibrium and body weight. The present investigations were directed toward that end.

METHODS

The animals employed were 84 healthy adult male albino rats (Sprague-Dawley) whose weights ranged from 283 to 353 gm at the start of the experiments.

The test rations used were identical with ration A of the previous papers of this series (Frazier et al., '47; Wissler et al., '48). In order to find the minimum maintenance level for a specific amino acid, a series of diets were constructed in which all constituents were maintained constant except the amino acid under consideration. Each diet contained a different quantity of this acid. On the first trial the quantities of the test acid were varied widely in an attempt to bracket the maintenance level. When a first approximation had been reached in this run, more levels were utilized to fill gaps in the original data, particularly in the region of the maintenance point. Each diet was fed to a single animal except in the case of phenylalanine, where three animals were used to test 9 different diets.

No additional nitrogen was added to replace that removed by the reduction in the quantity of the amino acid under investigation. The total nitrogen offered by 15 gm of diet therefore varied from 203 to 223 mg, of which 8.4 mg came from constituents of the diet other than the amino acids. All animals weighing up to 325 gm were offered 15 gm of diet per day, and those weighing 329 to 353 gm were offered 16 gm per day. This was done in order to maintain the caloric intake

level at or slightly above 1,200 Cal. per square meter of body surface per day and the nitrogen intake level at or slightly above 5.0 gm per square meter per day.

The diets all contained adequate minerals, vitamins, fat and roughage (see Frazier et al., '47; Wissler et al., '48). On arrival in the laboratory, the animals were housed in group cages and fed our stock 22% casein ration for a period of two to three months, until their weights were 290 to 350 gm. Then for several weeks prior to the feeding of the amino acid rations they were stabilized on a diet identical with ration A except that it contained 9% of its weight as bovine casein in place of the amino acid mixture. This diet provided 234 mg of nitrogen per 15 gm. For the last 5 or 7 days of the stabilizing period, nitrogen balance was determined. The casein was then replaced by an amino acid ration, which was fed for a period of 20 days. Diet consumption, fecal and urinary nitrogen and weight changes were determined for the first and second weeks of this period. During the last 6 days only diet consumption and weight were measured. The nitrogen balance estimations and other technical procedures have been described elsewhere (Wissler et al., '48).

OBSERVATIONS

In the preliminary stabilizing period on the casein ration all animals were in positive nitrogen balance. Weight changes in this period were either slightly negative or slightly positive, with a tendency on the average to small losses, of the order of 6 gm.

The data on the individual amino acids are summarized in table 1. Recorded there are the actual intake levels of the amino acids under consideration. These are in milligrams of amino acid per 100 cm^2 of body surface per day. Only utilizable amino acids were included in our studies (see Wissler et al., '48). Surface area was calculated using Lee's formula (Lee, '29). Also recorded are the diet consumptions as percentages of the total diet offered in the 14-day nitrogen

TABLE 1

Diet consumption, weight changes and nitrogen balances of adult well-nourished rats fed amino acid mixtures with varying quantities of individual essential amino acids

ANIMAL NUMBER	AMINO ACID INTAKE	DIET CONSUMED IN 14 DAYS	N BALANCE IN 14 DAYS	WEIGHT CHANGE IN 20 DAYS	ANIMAL NUMBER	AMINO ACID INTAKE	DIET CONSUMED IN 14 DAYS	N BALANCE IN 14 DAYS	WEIGHT CHANGE IN 20 DAYS
	mg/100 cm²/day	*%*	*mg*	*gm*		*mg/100 cm²/day*	*%*	*mg*	*gm*
		Histidine					*Lysine*		
83–1	0.0	81	— 157	— 46	85–2	0.0	97	— 234	— 23
89–3	1.1	86	— 257	— 33	298–3	2.0	96	— 105	+ 6
298–7	1.2	98	— 222	— 13	87–4	2.5	99	— 120	— 14
299–4	2.0	96	— 167	— 6	299–7	4.3	98	— 61	— 6
89–1	2.5	97	+ 320	+ 4	82–3	4.8	97	+ 404	+ 1
139–7	2.7	95	+ 256	+ 4	140–2	5.1	98	+ 9	0
88–6	3.7	98	+ 288	+ 9	139–3	5.5	97	+ 153	+ 6
		Tryptophan					*Threonine*		
90–3	0.0	97	— 500	— 54	88–2	0.0	99	— 835	— 42
88–7	1.0	94	— 300	— 44	88–3	1.3	96	— 228	— 23
88–4	1.8	98	+ 130	— 16	90–4	3.9	95	— 209	— 17
141–2	1.8	99	— 16	— 1	299–6	4.3	95	— 284	— 10
141–4	2.0	99	— 129	— 5	4–1	5.3	99	— 48	— 2
139–1	2.3	99	+ 301	+ 2	141–6	5.5	98	+ 234	+ 4
5–6	2.5	100	+ 553	+ 10	141–1	6.1	98	+ 217	— 3
87–5	2.5	98	+ 347	— 8	89–4	6.6	99	+ 277	+ 5
6–6	3.2	97	+ 546	+ 14					
		Methionine					*Leucine*		
87–6	0.0	99	— 448	— 22	82–1	0.0	86	— 178	— 48
82–7	1.3	99							

Methionine

87–6	0.0	99	— 448	— 22
82–7	1.3	99	— 392	— 33
90–1	3.9	99	— 43	— 23
82–2	6.5	99	+ 42	— 17
6–7	6.5	99	+ 79	— 4
3–1	7.8	99	+ 279	+ 11
141–3	7.8	99	+ 174	+ 4
2–7	9.1	99	+ 466	+ 6

Isoleucine

88–5	0.0	66	— 668	— 84
85–4	5.8	91	— 459	— 67
297–1	6.2	80	— 352	— 51
92–2	8.7	99	+ 49	— 24
92–7	8.8	97	— 90	— 17
86–5	8.9	99	— 412	— 39
87–1	11.0	99	— 52	— 24
299–5	11.5	96	+ 156	— 4
91–4	12.0	99	+ 468	— 20
6–2	13.2	96	+ 232	— 8
142–4	13.5	99	+ 522	+ 15
90–7	14.6	98	+ 408	— 13
1–3	16.4	98	+ 265	+ 13

Valine

85–3	0.0	74	— 624	— 48
86–4	5.0	98	— 138	— 25
85–7	7.6	99	— 42	— 11
139–4	9.8	98	+ 418	+ 8
139–6	10.1	90	+ 310	+ 5
84–2	10.2	99	+ 49	— 13
140–6	12.3	98	+ 516	+ 16
4–5	15.7	99	+ 415	+ 9

Leucine

82–1	0.0	86	— 178	— 48
297–2	2.7	87	— 253	— 34
298–4	5.3	84	+ 83	— 23
82–4	6.2	99	— 242	— 25
142–6	7.4	96	+ 278	+ 1
4–7	8.4	98	+ 261	+ 3
6–1	10.5	99	+ 322	+ 10
83–2	12.3	99	+ 399	+ 3
83–7	18.4	99	+ 399	— 5

Phenylalanine

5–5	0.0	89	— 284	— 28
51–3	1.2	58	— 158 [1]	— 34 [2]
52–3	1.7	52	— 288 [1]	— 57 [2]
5–7	2.4	93	— 233	— 22
51–2	3.1	72	— 17	— 22
52–3	3.4	63	+ 118 [1]	— 20 [2]
51–3	4.3	67	+ 88 [1]	— 20 [2]
2–6	4.8	94	+ 105	— 12
140–1	5.4	96	+ 133	— 4
139–2	6.0	97	+ 173	0
51–3	6.6	90	+ 128	+ 8
5–3	7.6	99	+ 300	0
52–3	7.9	98	+ 272	+ 22
51–2	9.2	100	+ 206	+ 20

[1] Calculated from a single 7-day N, balance.

[2] Calculated from a single 7-day weight change.

balance period, the net nitrogen balance in milligrams per 14 days, and the net weight change for the 20 days of amino acid feeding.

From the data in table 1 a graph was constructed for each amino acid examined. This was done by plotting the net nitrogen balance value against the amino acid intake level. A smoothed curve was drawn to the points and the level of intake necessary for maintenance of nitrogen equilibrium was estimated from the point at which the line intersected the ordinate

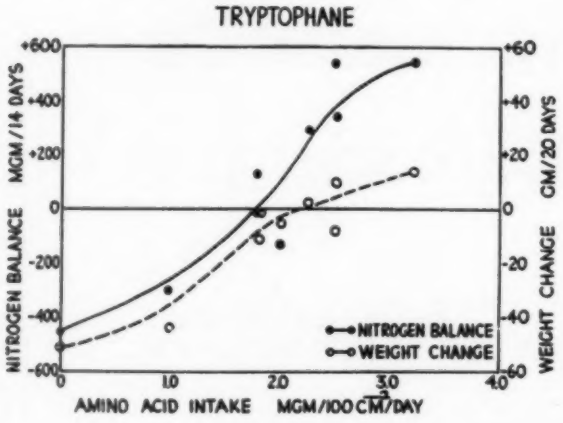

Figure 1

representing nitrogen equilibrium. A similar curve was constructed for weight changes versus amino acid intake. Figure 1 is a representative example of the curves obtained from the data on tryptophan.

It is evident from table 1 that with few exceptions diet consumption was excellent. The notable exceptions were when certain amino acids were omitted entirely from the diet, e. g., leucine, isoleucine, valine and phenylalanine. In addition there was some restriction of diet consumption at the lower levels of phenylalanine intake.

The data on fecal nitrogen excretion indicate complete absorption of the nitrogen of the amino acid ration. Thus the animals on the test rations containing amino acids excreted on the average 202 mg of fecal nitrogen per animal per week. Four animals receiving a protein-free diet excreted on the average 218 mg per week. Within the limits of experimental error this represents 100% absorption. Absorption of the nitrogen of the casein ration was only a little less, averaging 98%. No significant correlation was found between the level of intake of any of the amino acids and the fecal nitrogen

TABLE 2

Minimum essential amino acid requirements for maintenance of the adult rat

AMINO ACID [1]	ESTIMATE FROM NITROGEN BALANCE	ESTIMATE FROM WEIGHT CHANGE
	mg per 100 cm²	*mg per 100 cm²*
Tryptophan	1.8	2.2
Histidine	2.1	2.2
Phenylalanine	3.1	6.0
Lysine	3.7	4.5
Threonine	5.1	5.3
Methionine	5.3	7.3
Leucine	6.5	8.0
Valine	7.0	10.0
Isoleucine	12.9	13.7

[1] All values are given in terms of "utilizable" forms of the amino acids (see Frazier et al., '47).

excretion. Therefore the rather consistent increase in the nitrogen balance values (table 1) with increasing intake levels of each of the amino acids tested reflects largely the reduction in the urinary nitrogen excretion.

The minimum maintenance levels derived from these experiments are recorded in table 2. Estimates based on both the nitrogen balance and the weight balance are shown. Very reasonable agreement between the two methods of estimation is apparent except in the case of phenylalanine, where the quantity estimated from the weight balance is approximately twice that estimated from nitrogen equilibrium. It can be seen

from the data on this amino acid (table 1) that diet consumption was poor at the lower levels of intake. This may have influenced the weight gain but seems to have had much less effect upon the attainment of nitrogen equilibrium. It is evident that for the other amino acids also the estimates from the weight balance are consistently somewhat higher than from the nitrogen balance. Two factors may be operating here. As was observed in the preliminary period on the casein diet, there is some dissociation between nitrogen metabolism and weight gain. With large animals the capacity of the fat and fluid compartments of the body to vary independently of the principal nitrogen-containing compartment, protein, appears to be appreciable and much greater than that observed with smaller depleted animals (Steffee et al., '50). It was for this reason that the nitrogen balance method was used in these experiments. The other factor is a technical one. A rat urinates in small quantities. Even when the animal is housed on a wide mesh wire screen, some of the urine clings to the wire screen and dries there with a loss of ammonia or is rubbed off onto the animal's fur. Such unavoidable losses reduce the apparent nitrogen excretion and consequently increase the apparent nitrogen balance. Such errors do not seem to be large and appear to be consistent under a uniform set of conditions.

Despite the presence of a consistent discrepancy between the estimates of the minimum essential amino acid requirements derived from weight and from nitrogen balance data, the observations appear valid. Table 2 demonstrates that the two sets of observations actually substantiate each other, since they give almost identical orders of rank for the 9 amino acids, the only exception being phenylalanine.

In order to test the validity of the estimates for the 9 essential amino acids, two diets were constructed. In diet I the level of each of the essential amino acids was that estimated as the minimum necessary for maintenance of nitrogen equilibrium, and in diet II the minimum necessary for maintenance of weight equilibrium. Each of these diets was fed to a pair

of animals prepared in the same fashion as for the original estimations of requirements. Nitrogen balance, diet consumption and weight changes were recorded for a period of two weeks. The results are presented in table 3. One of the two animals on diet I remained in positive balance for the two weeks, the other was in slightly negative balance. Both animals on diet II were in postive balance for both periods. All animals showed a moderate weight loss.

TABLE 3

The effect of feeding amino acid rations containing the essential amino acids at the minimum levels estimated to be necessary for maintenance

ANIMAL NUMBER	DIET [1]	NITROGEN				BODY WEIGHT	
		Intake	Output		Balance	At start of period	Change
			Urinary	Fecal			
		mg	*mg*	*mg*	*mg*	*gm*	*gm*
92–4	9% Casein	1,700	844	295	+ 561	328	+ 7
	I	963	666	251	+ 46	335	— 5
	I	957	682	222	+ 53	330	— 7
91–7	9% Casein	1,605	896	292	+ 417	310	0
	I	909	724	244	— 59	310	— 6
	I	913	724	209	— 20	304	— 11
90–1	9% Casein	1,610	925	265	+ 420	305	+ 2
	II	956	687	220	+ 49	307	— 3
	II	980	673	211	+ 96	304	— 7
91–2	9% Casein	1,590	801	276	+ 513	296	+ 5
	II	935	705	227	+ 3	301	— 2
	II	924	639	211	+ 74	299	— 11

[1] Each diet period of 7 days' duration. Diet I contained the minimum quantity of essential amino acids for maintenance estimated from *nitrogen* equilibrium; diet II the same, as estimated from *weight* equilibrium.

In addition to the balance points on the curves, the form of the curves is of interest. Figure 1, the curve for tryptophan, demonstrates the general character of all of the curves for nitrogen balance; they tend to have an "S" shape. From previous work (Allison and Anderson, '45; Benditt, Woolridge and Stepto, '48) it might be expected that in the region of negative nitrogen balance there would be a linear relationship between the amino acid intake level and the nitrogen

balance. This is not the case, and even in the complete absence of any one of the amino acids, with the possible exception of threonine, there is diminution in nitrogen excretion.

In order to test this point further, nitrogen excretion was determined on 4 animals fed a protein-free diet for a period of 14 days. The average weight of these animals was 306 gm; they were prepared in the same manner as the others used for testing the amino acid levels, and fed a diet isocaloric with and of composition identical to the other test diets except that the amino acid or protein source was omitted and replaced by a calorically equivalent quantity of carbohydrate. These animals showed a net balance of -990 mg of nitrogen in the 14 days, with a standard deviation of ± 74 mg. Comparing this value with net nitrogen balance values for the animals lacking one essential amino acid (table 1) it is evident that with the exception of the rats lacking threonine all of the latter excreted significantly less nitrogen than the animals on the protein-free diet. Certain incomplete amino acid mixtures therefore appear to exert some nitrogen sparing action in the rat.

DISCUSSION

The present observations substantiate those previously reported by us on the indispensability of 9 of the so-called essential amino acids for the maintenance of weight and nitrogen equilibrium in the adult rat. They demonstrate that not only is it impossible for the rat to achieve nitrogen equilibrium in the complete absence of any one of these 9 amino acids (tryptophan, lysine, histidine, methionine, threonine, phenylalanine, valine, leucine, and isoleucine), but that a certain minimum quantity of each of these acids is necessary for the attainment of nitrogen and of weight equilibrium. In broad terms, then, in order to maintain the "continuing metabolism" of an animal and consequently a normal state of health and well-being, there is demanded a full quantitative as well as qualitative complement of the essential amino acids.

The fact that diets I and II, containing the essential amino acids in the minimum quantities, maintained nitrogen equilibrium in 3 out of the 4 animals in which they were tested, is of some importance. It supports the validity of the estimates made of the individual amino acid requirements. Furthermore, these diets contained less than two-thirds of the total nitrogen present in the standard amino acid ration (ration A) used in this laboratory, indicating that the most economical means of supplying the maintenance nitrogen needs of an animal is by the use of a mixture which contains the amino acids, at least the essential ones, in the proportions in which they are used by the animal body.

The amino acid requirements reported here were determined under a standard set of conditions, including an adequate caloric intake, sufficient vitamins and minerals, a fixed proportion of the caloric intake as fat, and amounts of the non-essential amino acids such as might be supplied by a natural protein (e. g., casein). Certain of the non-essential amino acids were not in the mixture (serine, norleucine, proline and hydroxyproline). These and other conditions may operate to vary the apparent requirement of any one or all of the essential amino acids for maintenance. Therefore it is best to consider the levels given here as first approximations.

The sparing action of non-essential amino acids on essential amino acids has already been demonstrated for certain amino acid pairs. Thus, tyrosine in sufficient quantity can reduce the phenylalanine requirement by about one-half (Womack and Rose, '46), and a sufficient quantity of cystine can reduce the methionine requirement by about one-sixth (Womack and Rose, '41). The diets used in the present investigations probably contained sufficient tyrosine to reduce the phenylalanine requirement for synthesis of tyrosine to its minimum. The quantity of cystine, similarly, was probably sufficient to reduce the methionine requirement for conversion to this acid nearly to its minimum. In the case of lysine, which appears (Schoenheimer, '42) to be unique in not entering into cross reactions with other amino acids, we are probably dealing with a fairly

close approximation to the minimum requirement for maintenance. We cannot be certain that this is so in the case of the other amino acids.

The various functions of the essential as well as the non-essential amino acids largely remain to be investigated. In the cases of growth and rehabilitation, the relationship between the dietary requirement of the amino acids and their ultimate use appears relatively clear. Tissue construction appears to be their principal function, and, as might be expected, the relative quantities demanded by the organism for optimum utilization of a mixture of amino acids approximate those found in the tissues which constitute the bulk of the organism (Cannon, '48). With respect to maintenance, a more complex relationship appears to exist. Maintenance represents a steady state in which not only is there tissue breakdown and replacement, but detoxification and other normal metabolic processes occur in which amino acids are intimately involved. It is possible that in the equilibrium state the non-structural functions of certain amino acids may be of comparable or even greater magnitude than their structural functions. The observation that imcomplete amino acid mixtures as well as single amino acids will spare body tissue (Brush, Willman and Swanson, '47), which we have in part confirmed here, lends credence to this idea. The above workers found that the sum of the nitrogen spared by the individual essential amino acids almost exactly accounted for the total nitrogen loss on a protein-free diet. This observation needs confirmation. If true, it would appear to indicate that the functions of the amino acids in maintenance are almost wholly non-structural in the usual sense of the word. Furthermore it would seem to indicate that the nitrogen loss on a protein-free diet is due to the utilization of certain amino acids obtained from the continuous hydrolysis of tissue protein. The remaining incomplete mixture of amino acids is then discarded, since such a mixture cannot be used for synthesis of complete proteins.

It is of some interest to compare the apparent minimum amino acid requirements for maintenance of our adult well-nourished rats with those estimates now available for man. Harte and Travers ('47) have computed some estimates from published data on whole proteins of varying amino acid composition. Rose ('49), using purified amino acid mixtures, determined the requirements for maintenance of nitrogen equilibrium in healthy young adult males. In order to compare the values for the rat with the estimates for man the

TABLE 4

Comparison of minimum essential amino acid requirement for maintenance of nitrogen equilibrium in adult rats and in men

AMINO ACID	REQUIREMENTS FOR MAN ESTIMATED FROM REQUIREMENTS FOR N_2 EQUILIBRIUM IN THE RAT	REQUIREMENTS FOR MAN (HARTE AND TRAVERS, '47)	REQUIREMENTS FOR MAN (ROSE, '49)
	gm	*gm*	*gm*
Tryptophan	0.27	0.4	0.25
Histidine	0.31	0.5	...
Phenylalanine	0.47	1.4	1.10
Lysine	0.56	0.8	0.80
Threonine	0.76	1.0	0.50
Methionine	0.79	0.5	1.10
Leucine	0.97	1.7	1.10
Valine	1.05	1.1	0.80
Isoleucine	1.68	1.2	0.70

assumption was made that continuing nitrogen metabolism and hence the maintenance amino acid requirements are linearly related to the body surface area or some approximate measure thereof (Brody, '45). The values for the rat were extrapolated on this basis to a surface area of 1.5 m^2.

If one considers the differences in the conditions employed in arriving at the three sets of minimum amino acid requirements presented in table 4, the agreement among them is rather surprising. Only two gross discrepancies are apparent. The phenylalanine requirement as estimated from the rat data is quite low and the isoleucine estimate is rather high com-

pared with the estimates from observations on humans. All three values for the tryptophan requirement are quite close, and demonstrate that this amino acid is required in the smallest quantity. It is a little surprising that Rose's estimate for methionine is so high in comparison with the others, since there is recent evidence (Johnson et al., '47; Cox et al., '47) that the methionine requirement of man is relatively smaller than that of the rat (Brush, Willman and Swanson, '47) and the dog (Allison, Anderson and Seeley, '47).

SUMMARY AND CONCLUSIONS

Quantitative requirements for the 9 amino acids indispensable for maintenance of weight, appetite and nitrogen equilibrium of the adult well-nourished male albino rat have been measured. The measurements were made under a standardized set of conditions, including adequate caloric, vitamin and mineral intakes. Total nitrogen intake was more than sufficient to supply the maintenance needs of the animals. The level of the amino acid under consideration was varied, while all other factors of the diet were maintained constant. Weight changes and nitrogen balance were determined. The minimum quantity of each amino acid necessary to maintain nitrogen equilibrium and weight balance was estimated from the curves relating amino acid intake to nitrogen balance or weight change, respectively. From the data presented the following conclusions were drawn:

1. The findings substantiate those previously reported from this laboratory that tryptophan, lysine, histidine, threonine, methionine, valine, phenylalanine, leucine and isoleucine are all essential for the maintenance of weight and nitrogen equilibrium in the adult well-nourished rat, since in the complete absence of, or in the presence of an insufficient quantity of, any one of these amino acids neither nitrogen equilibrium nor weight was maintained.

2. The minimum levels for each of the above-mentioned amino acids necessary for the maintenance of weight and nitrogen equilibrium under standard conditions are presented.

3. Mixtures of amino acids lacking an essential amino acid, while they cannot establish nitrogen or weight equilibrium, do have some nitrogen sparing action.

4. Reasons for believing that the maintenance functions of the amino acids differ in certain important respects from their functions in growth or in the rehabilitation of the depleted organism are briefly discussed.

5. A comparison is made between the minimum essential amino acid requirements for man as found in the literature and for the rat as derived from the present experiments. There is reasonable agreement between the two sets of estimates. This suggests once more that there is a fundamental similarity among the mammalian organisms, and reaffirms the value of lessons learned from the animals for use as guides in human nutritional planning and studies.

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THE EFFECT OF FAT LEVEL OF THE DIET ON GENERAL NUTRITION

V. THE RELATIONSHIP OF THE LINOLEIC ACID REQUIREMENT TO OPTIMUM FAT LEVEL [1,2]

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FOUR FIGURES

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Although fat has long been regarded as an optional component of the diet and one which is interchangeable with carbohydrate, a number of recent reports have indicated that it probably should be classed as an essential foodstuff. The role played by fat in the diet cannot entirely be explained by the essential fatty acids which it supplies, but must be traced to some additional factor or factors.

In earlier experiments in this laboratory it was found that rats grew best when fats comprised 20 to 40% of the diet, which corresponds with 38 to 62% of the total calories (Deuel et al., '47). Better reproductive performance and lactation and greater physical capacity were also noted on the high fat

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We are indebted to Merck and Company for the α-tocopherol.

[2] The results reported here were presented at the Ninth Annual Convention of the Institute of Food Technologists in San Francisco, July 9–14, 1949.

diets. When animals were subjected to a sufficient restriction in calories so that only minimum growth obtained during a 12-week period following weaning, subsequent growth was best in those rats on the high-fat regime when ad libitum feeding was instituted (Scheer et al., '47b). In fact, marked growth was still a characteristic of the rats on the 10, 20, and 40% fat levels during the 9- to 12-week period of recovery, while it had practically stopped by that period in those rats on the 0 and 5% fat levels. In later tests on young adult rats subjected to caloric restriction with a resultant marked drop in body weight, it was again found that weight loss was less rapid, mortality was less, recovery on reinstitution of ad libitum feeding was quicker, and reproductive capacity was superior in rats receiving diets containing fats (Scheer et al., '47a). Scheer et al. ('47c) demonstrated that, although the fat content of rat carcasses was slightly higher on high-fat diets than on diets low in fat, the increased body weight cannot be accounted for on the basis of fat alone and that, in fact, the fat content of the body and that of the diet are not quantitatively related.

The beneficial effects of high fat diets on the growth of rats reported from this laboratory confirm earlier studies of other workers. Thus, an increase in the fat content of isocaloric diets from 2% to 30% resulted in a greater weight increase as well as in a decrease in heat production (Forbes, Swift, Elliott and James, '46a; Forbes et al., '46b). More recently, French, Black and Swift ('48) found that rats on a low-protein regime made more rapid and efficient gains in weight on a 30% fat diet than when only 2% of fat was included. Finally, Pearson and Panzer ('49) have reported the growth of rats fed ad libitum diets containing 8% of corn oil or lard to be 29% superior to that of animals which received no added fat but were supplemented with ethyl linoleate. However, the latter investigators noted no significant differences in the gain of the rats when the intake of protein and energy was equalized. On the other hand, a more efficient utilization of the calories on high-fat diets might well be expected from the fact that the specific

dynamic effect of protein and fat is not additive, as it is with protein and carbohydrate (Forbes and Swift, '44).

The finding that an increased work capacity occurs in rats on high fat diets (Deuel et al., '47) was unexpected in view of the usual opinion that carbohydrate affords the most effective fuel for muscle work. Samuels and co-workers ('48) have recently confirmed the earlier results of Deuel et al. ('47) by the demonstration that rats fed on diets containing 80% of the calories as fat for 45 days survived longer and accomplished a significantly greater amount of work during a subsequent period of fasting than animals which had received diets containing a similar proportion of carbohydrate or protein. Gilmore and Samuels ('49) believe that a different metabolic picture obtains in the muscles of the fat-fed group than in those of the carbohydrate-fed animals. Glucose utilization and glycogen disappearance from the diaphragms of the former group were much lower than for those of the latter series. These workers confirm the results of Lundbaek and Stevenson ('48) in so far as the effect on glucose utilization is concerned.

Another feature of the metabolism of fats is their protein-sparing action. Although no changes were noted by Willman et al. ('47) in the urinary nitrogen in rats receiving protein-free diets containing no fat or 20% of fat when the caloric intake was 100 or 75% of that required, the excretion of urinary nitrogen was maintained at the low level at the 50% caloric intake only when the diet containing 20% of fat was fed. The negative nitrogen balance when the protein-free diets were given at the 25% caloric requirement was considerably less with the fat diet than with the one from which this foodstuff was absent. Somewhat similar results have been reported by Schwimmer and McGavack ('48) for man. In connection with these studies, the recent report of Pearson and Panzer ('49) is of considerable interest. These workers have reported that the fecal and urinary excretion of phenylalanine, lysine, valine and methionine were significantly less for rats receiving corn oil than for those whose diets contained no added fat. This variation could not be ascribed to a lack

of the essential fatty acids, as the rats in both groups received a supplement of ethyl linoleate each day sufficient to meet daily requirements.

There are other circumstances in which fat seems to fill the metabolic needs more satisfactorily than carbohydrate. Dugal and co-workers ('45) found that the survival of rats kept at an environmental temperature of —4°C. on a high-fat diet was substantially greater than the survival of those on a low-fat regime. It was demonstrated that at these low temperatures the rats voluntarily selected a diet containing as much as 50% of the calories in the form of fat, while a lower fat content was preferred at higher environmental temperatures.

Since the pioneer work of Evans and Lepkovsky ('29) and others (Arnold and Elvehjem, '39; Stirn et al., '39), it has been recognized that fat exerts a thiamine-sparing action. A relationship between the requirement for pyridoxine and the amount of the essential fatty acids in the diet was first demonstrated by Birch ('38) and has been confirmed by a number of other workers (Schneider et al., '40; Salmon, '41; Quackenbush et al., '42). On the other hand, oleic acid may cause a growth inhibition on diets containing suboptimum amounts of pyridoxal or pyridoxamine (Sarma et al., '47). Conversely, oleic acid has been shown to replace biotin in the bioassay with *Lactobacillus casei* (Williams and Fieger, '46, '47), especially when used in connection with a non-fatty surface-active agent such as serum albumin (Williams and Fieger, '49). Finally, fats are of special merit because they contribute the essential fatty acids — linoleic, linolenic and especially arachidonic — which are required for the satisfactory functioning of the tissues. The wide literature on the essential fatty acids has been summarized by Burr ('42).

From the above discussion it would appear that the metabolism of fats is connected, directly or indirectly, with that of protein and some of the water-soluble vitamins. The behavior of fat under such conditions certainly cannot be explained merely on the basis of its being a source of calories.

The present experiments were undertaken in order to determine by new procedures whether fat contributes to nutrition other than as a source of the essential unsaturated acids and as a reservoir for meeting caloric needs. Since it had been demonstrated earlier (Ershoff and Deuel, '45) that deficiencies in diet could be accentuated by the administration of the growth hormone, an attempt was made to accelerate the appearance of fat deficiency symptoms by the use of this hormone.

TABLE 1

The composition of the diets employed in the experiments

DIETARY COMPONENT	DIET 69, FAT-FREE	DIET 70, CONTROL DIET
	%	%
Casein extracted [1] or vitamin-test [2]	20	20
Sucrose	71.67	61.67
Cottonseed oil [3]	. .	10
Cellulose [4]	4	4
Salt mixture [5]	4	4
Water-soluble vitamin mixture [6]	0.33	0.33

[1] Vitamin-test casein further extracted by a variety of procedures. See text.

[2] Vitamin-test casein from General Biochemicals, Inc.

[3] Wesson oil.

[4] Cellu flour obtained from the Chicago Dietetic Supply House.

[5] Osborne-Mendel salt mixture.

[6] The water-soluble vitamin mixture had the following percentage composition: choline and inositol, each 36.70; para-aminobenzoic acid, 18.35; thiamine chloride hydrochloride, 2.20; calcium pantothenate, 2.06; nicotinic acid, 1.83; riboflavin and pyridoxine hydrochloride, each 0.83; folic acid, 0.31; 2 methylnaphthoquinone, 0.15; and biotin, 0.06.

METHODS

The composition of the diets is given in table 1.

Supplements of the fat-soluble vitamins dissolved in ethyl alcohol were administered orally by syringe three times weekly. The daily dosage was as follows: vitamin A, 21 U.S.P. units; vitamin D_3, 2.1 U.S.P. units; α-tocopherol, 0.11 mg.

Although Brown et al. ('38) demonstrated that the rigid exclusion of fat from the diet described earlier by Burr and Burr ('29) is probably not necessary for the production of the fat-deficiency syndrome, the original Burr diet was employed,

using a mixture of synthetic B vitamins in place of extracted yeast. The casein used was vitamin-test casein unextracted, extracted 7 days with a mixture of diethyl ether and alcohol, dissolved and precipitated only, or extracted 3 or 7 days with diethyl ether and alcohol following solution and reprecipitation. Since no differences were noted in any case within the 12-week period required for depletion and to reach a plateau in body weight, the depleted animals from the various groups were assigned at random to the different subsequent tests on the fat-deficient rats.

The rats used were weanling rats obtained from our own colony. The mothers were kept on a low-fat diet subsequent to the birth of the litters. The young rats were assigned at weaning to the several depletion groups and to the positive control group (group V). Parallel tests were made on male and female rats.

After 12 weeks on the several depletion diets the following groups were assembled: Group I, negative control group, which was continued on the fat-free regime; group II, similar to group I except that 150 mg of growth hormone were injected daily; group III, diet similar to group I but supplemented with 20 mg of methyl linoleate daily; group IV, diet similar to group I but supplemented with 20 mg of methyl linoleate daily and also injected with 150 mg of growth hormone each day; group V, which was continued on the 10% fat diet (diet 70).

The negative control groups with and without the growth hormone were discontinued after 14 weeks. After 22 weeks, when the effect of the linoleate had largely worn off, the supplement was increased from 20 to 60 mg daily in groups III and IV. When this increase in linoleate intake was shown to result in no augmentation in growth, the animals were transferred to control diet 70 on which they were continued for 7 weeks, at which time the experiment was terminated. Because of the exhaustion of our supply of the growth hormone, injections of the hormone were stopped at the end of the linoleate administration (25th week).

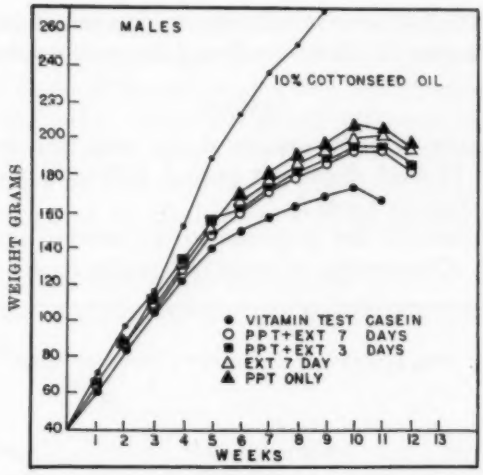

Fig. 1 The average weights of weanling male rats receiving fat-free diets containing casein extracted by various procedures, and of a group of rats receiving a similar diet which contained 10% of cottonseed oil.

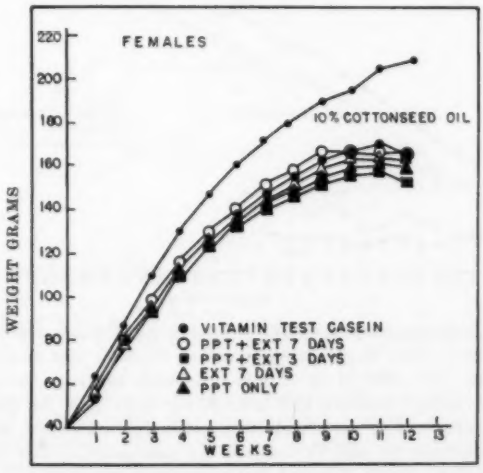

Fig. 2 The average weights of weanling female rats receiving fat-free diets containing casein extracted by various procedures, and of a group of rats on a similar diet which contained 10% of cottonseed oil.

The growth hormone employed was a commercial preparation.[3] The methyl linoleate used was the pure product.[4]

RESULTS

The changes in body weight of the male and female rats during the 12-week depletion period following weaning are included in figures 1 and 2.

No variations in the response to the several preparations were noted. Commercial vitamin-test casein is as satisfactory

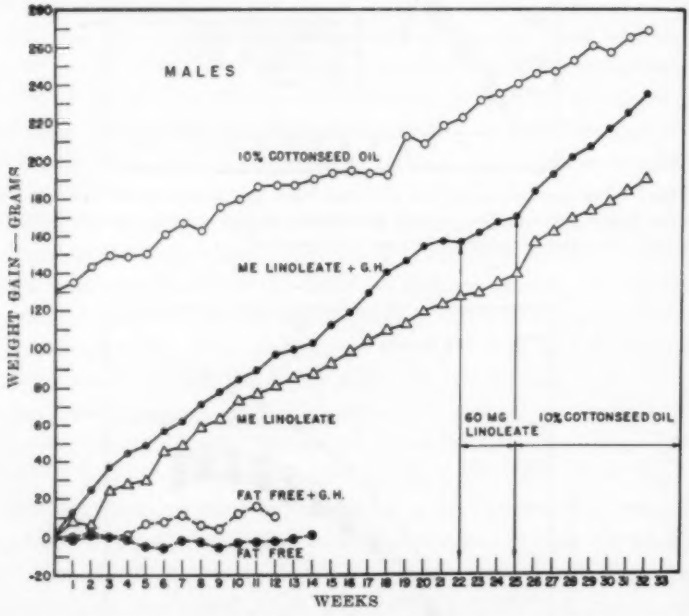

Fig. 3 The average gains in weight of male rats previously receiving a fat-free diet until depleted, when they were continued on the same diet alone (I), with the growth hormone (II), with 20 mg of methyl linoleate and later 60 mg daily (III), with 20 mg of methyl linoleate and later 60 mg daily plus the growth hormone (IV), or receiving a diet containing 10% cottonseed oil before and throughout the test (V). Groups III and IV received the cottonseed oil diet during the last 7 weeks without administered growth hormone.

[3] Kindly furnished us by D. I. M. Bunding of Armour and Co.

[4] Supplied by the Hormel Foundation, Austin, Minnesota.

for such studies as are samples which have been reprecipitated or exhaustively extracted, or both. These results are in agreement with those of Brown et al. ('38), who suggest that even commercial casein can be employed, although the depletion period in this event is somewhat lengthened. However, in the present series no variation in the time required to effect depletion was noted on further purification of the vitamin-test casein.

The weight changes of the rats placed on the several experimental regimes after depletion are pictured in figures 3 and 4.

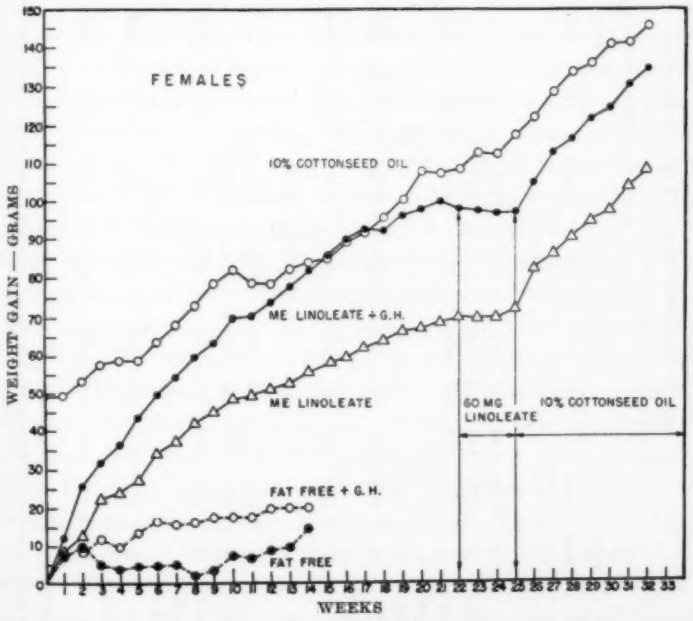

Fig. 4 The average gain in weight of female rats previously receiving a fat-free diet until depleted, when they were continued on the same diet alone (I), with the growth hormone (II), with 20 mg of methyl linoleate and later 60 mg daily (III), with 20 mg of methyl linoleate and later 60 mg daily plus the growth hormone (IV), or receiving a diet containing 10% cottonseed oil before and throughout the test (V). Groups III and IV received the cottonseed oil diet during the last 7 weeks without administered growth hormone.

TABLE 2

The average body weight and survival of male and female rats previously on a fat-free diet until depleted and thereafter receiving no supplement, the growth hormone alone, methyl linoleate alone or with the growth hormone along with the fat-free diet, and of a control group of rats raised and continuing on a similar diet containing 10% of cottonseed oil

GROUP NO.	DIETARY SUPPLEMENT OR TREATMENT	NO. OF RATS AT START	AVERAGE BODY WEIGHT IN GRAMS ON FOLLOWING WEEKS [1]											
			Period 1						Period 2 [2,3]			Period 3 [2,4]		
			Start	1	2	6	10	14	18	22–0	25–3	26–1	29–4	32–7
			Male rats											
I	None	5	186	184	187	174	176	191(4)						
II	Growth hormone	5	182	185	189	198	203(4)	223(3)						
III	Methyl linoleate	5	174	182	181	220	247	261	284	302	314	331	338	366
IV	Methyl linoleate and growth hormone	6	173	186	198	230	258	277	313(5)	329(5)	342(5)	356(5)	380(5)	408(5)
V	Control diet with 10% fat	5	304	309	317	334	353	363	366	396	414	419	434	442
			Female rats											
I	None	6	158	166	168	163	165	172(5)						
II	Growth hormone	6	160	168	169	175(5)	176(5)	178(5)						
III	Methyl linoleate	6	157	166	170	191	206	213	221	227	230	239	252	265
IV	Methyl linoleate and growth hormone	6	161	174	187	202	231	243	253	259	258	266	285	296
V	Control diet with 10% fat	5	207	207	210	222	239	241	253	265	275	279	293	303

[1] The figures in parentheses indicate the number of rats remaining alive at that period.

[2] The first figure in each column heading gives the total number of weeks on experiment, while the second figure gives the weeks on period 2 or 3.

[3] Groups III and IV received 60 mg of linoleic acid instead of 20 mg daily.

[4] Groups III and IV received the diet containing 10% cottonseed oil during this period in addition to group V, which received the same diet throughout. The growth hormone was stopped at end of 25th week.

The starting weight for the control male and female rats was approximately 130 and 50 gm, respectively, above that for the depleted rats, which represents the average differences in weight at that time. Additional records of the experiments are included in table 2.

DISCUSSION

The depletion period, which lasted 12 weeks after weaning, was about the same as that reported by Burr and Burr ('29) and by Brown et al. ('38) for a less rigid diet. The further precipitation of the casein or its prolonged extraction with ethyl alcohol and diethyl ether, or both procedures, failed to shorten the depletion time. The diets were in general similar to those of Burr and Burr ('29) except that a mixture of pure synthetic B vitamins was used instead of ether-extracted yeast. The fat deficiency symptoms which appeared were eczematous lesions of tail and feet; they corresponded with the classical symptoms of this condition. After growth had ceased on the fat-free diet the animals remained at a uniform weight for 14 weeks, at which time the experiment was terminated. These results are further proof of the fat deficiency of our rats.

The administration of the growth hormone to the animals on the fat-free diet did not result in any appreciable growth. On the other hand, no noticeable rise in mortality occurred over that of the group which did not receive the injections of hormone. In this respect the action of the growth hormone differs from its behavior in vitamin A deficiency, where the survival time is markedly shortened (Ershoff and Deuel, '45). This discrepancy in results may indicate that the growth hormone at the level injected cannot exaggerate the symptoms of fat deficiency, or it may be traceable to the use of different preparations; the earlier preparation was one furnished by Dr. O. Kamm of Parke, Davis and Company, while the present sample was prepared by Armour and Company. However, about the same excess growth was obtained in the female rats receiving the growth hormone plus linoleate over that obtained

the same diet throughout. The growth hormone was stopped at end of 25th week.

in those rats supplemented only with linoleate as was observed with the earlier growth hormone preparation (Deuel et al., '46).

When the growth hormone is administered to rats previously depleted on fat-free diets but which are also receiving linoleic acid, the stimulation in growth is an immediate one which precedes by several weeks that caused by the linoleic acid alone. The two curves run parallel thereafter for approximately 32 weeks. In earlier tests in this laboratory it was found that the growth hormone was able to stimulate the growth of rats on diets which contained corn, cottonseed, peanut, or soybean oils, or butterfat or margarine fat (Deuel, Hendrick and Crockett, '46). Whether this stimulating effect of the growth hormone is ascribable only to the presence of the essential unsaturated acids or whether oleic acid, the saturated acids, or the non-saponifiable residue contribute to it is not indicated. However, the male animals receiving the growth hormone plus linoleic acid did not reach the body weights of the control rats on the 10% cottonseed diets. In the case of the female rats, these weights were attained for several weeks but could not be maintained. Had group V also received the growth hormone for a period corresponding with group IV, it is reasonable to postulate that the linoleate-supplemented, growth hormone injected rats would not have attained the body weights of the rats on the control (10% fat) diet also receiving the growth hormone.

Present tests indicate that the linoleate requirement of female rats on a fat-free diet is lower than 20 mg per day. The data are not so clear-cut in the case of male rats. The administration of linoleate at a level of 60 mg daily did not change the slow rate of increase in body weight of the male rats on the 20 mg dose, nor did it result in a stimulation in growth of the female rats which had reached a plateau on the 20 mg level. Martin ('39), who has carried out an extensive series of tests on the linoleic acid requirement of rats, has arrived at the 20 mg figure; also Burr and Burr ('29) and MacKenzie et al. ('39) are agreed that a daily dose of 20 mg of this acid is

sufficient to allow normal growth. Burr ('42) has suggested that a higher requirement may obtain on high-fat diets.

The marked acceleration in growth which resulted in groups III and IV when they were transferred from the fat-free, linoleate-supplemented diets to one containing 10% of cottonseed oil would seem to indicate that fat is important in growth other than as a source of the essential fatty acids. Although the intake of the essential fatty acids increased to 400 to 500 mg daily on the 10% cottonseed oil diet, a stimulation of growth should have been noted when the linoleate intake was raised from 20 to 60 mg had this been the limiting factor. It is possible that the greater efficiency of fat utilization over that of carbohydrate may have accounted for the sharp rise in growth. Another possibility is that some component present in the fat may have been responsible, although the known essential additional component (α-tocopherol) was given as a supplement. The possibility that the fat diet may have been more palatable than the low-fat regime, and so resulted in increased food consumption, is not precluded.

SUMMARY

1. Weanling rats fed a diet containing vitamin-test casein as such, or casein after reprecipitation or exhaustive extraction, or both, as well as sucrose, cellu flour, a salt mixture, and the synthetic B vitamins, developed symptoms of fat deficiency in 12 weeks.

2. The injection of the growth hormone in such fat-deficient rats did not result in growth but also did not cause death. In this respect the action of the growth hormone differs from that in vitamin A-deficient rats.

3. When 20 mg of linoleic acid were given as a supplement to the fat-deficient rats, a prompt response in growth resulted, which was not further augmented when the level was increased to 60 mg per day.

4. The injection of the growth hormone combined with the feeding of methyl linoleate to fat-deficient rats resulted in an immediate growth response, in contrast to the more tardy ac-

tion of the linoleate alone. Although the average body weight of the growth hormone-treated, linoleate-fed rats exceeded that of the animals receiving only the linoleate, the growth curves remained parallel throughout the test.

5. The administration of a 10% cottonseed oil diet to rats receiving linoleate at an optimum level resulted in further acceleration of growth. This may indicate that the oil possesses some effect other than that of linoleic acid; perhaps the results were dependent on an additive effect of other unsaturated acids or of fat itself.

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VITAMIN E CONTENT OF FOODS [1]

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Information regarding the vitamin E content of a variety of American foods has not been available. Consequently, we are presenting results obtained in our laboratory in the past 4 years, during which time we have analyzed commonly used foods for total tocopherols and for α-tocopherol, utilizing chemical procedures recently described (Quaife and Harris, '48). Incidentally, using these new values for vitamin E in foods, we have made an estimate of the average per capita consumption of total tocopherols and of α-tocopherol in the United States.

METHOD

The foods in this survey were purchased on the open market in Rochester, New York. Except for bakery products, they were analyzed in their raw state, following such treatment as would be given them in the home prior to cooking, i.e., washing, peeling, and removal of inedible portions.

The chemical procedures used for determining total tocopherols and γ- plus δ-tocopherols in plant and animal tissues have been described in detail by Quaife and coworkers ('48, '49). The difference between total and γ- plus δ-tocopherol values was used as the measure of α-tocopherol content except in wheat products, where β-tocopherol is known to occur. Values reported are the means of quadruplicate analyses.

[1] Communication no. 164.

RESULTS

Fruits

It is interesting to note (table 1) that essentially all of the vitamin E in those fruits tested occurs as α-tocopherol. Practically no γ- or δ-tocopherol was found. However, the quantity

TABLE 1

Tocopherol content of fruits and vegetables

FOOD	TOCOPHEROL CONTENT					
	mg/100 gm fresh material			% of total tocopherols	mg/gm extracted lipid	
	Total	γ + δ	α	α	*Total*	α
Fruits:						
Apples	0.74	< 0.02	0.72	> 97	3.3	3.2
Bananas	0.40	< 0.03	0.37	> 93	2.3	2.1
Grapefruit	0.26	< 0.01	0.25	> 96	1.6	1.5
Oranges	0.24	< 0.01	0.23	> 96	1.5	1.4
Vegetables:						
Beans, dried navy	3.60	3.50	0.10	3	1.5	0.1
Cabbage	0.11	0.05	0.06	55	0.7	0.4
Carrots	0.45	0.00	0.45	100	2.1	2.1
Celery	0.48	< 0.02	0.46	> 96	5.4	5.2
Lettuce, head 1	0.43	0.14	0.29	67	2.6	1.7
Lettuce, head 2	0.54	0.25	0.29	54	..	..
Onions	0.26	0.05	0.21	81	3.6	2.9
Peas, green	2.10	2.00	0.10	5	2.6	0.1
Potatoes, white, peeled	0.06	...	...	..	1.2	..
Potatoes, sweet	4.0	0.0	4.0	100	5.7	5.7
Tomatoes	0.36	0.09	0.27	75	6.7	5.0
Turnip greens	2.30	< 0.06	2.24	> 97	4.2	4.1

of α-tocopherol in an average serving of these fruits is quite low, 0.23 to 0.72 mg, and this category of foods must be rated as a rather poor source of vitamin E.

Vegetables

In generalizing about the total tocopherol content of the vegetables shown in table 1, one would consider that they are better sources of vitamin E than fruits, since they range in

concentration up to 2.30 mg per 100 gm for turnip greens and 3.60 mg for dried navy beans. However, with regard to their α-tocopherol content, most vegetables fall in the range from 0.06 to 0.45 mg per 100 gm, and are thus similar to fruits as sources of vitamin E activity. An exception is turnip greens, which appear to be an excellent vegetable source of vitamin E, with 2.24 mg of α-tocopherol per 100 gm.

The tocopherols of certain vegetables — turnip greens, carrots, and celery — are almost 100% in the α-form. The tocopherols in legumes, navy beans and peas, contain practically no α-tocopherol, while in other vegetables α-tocopherol represents from 50 to 80% of the total tocopherols.

It is interesting to note that the α-tocopherol content of the present samples of celery, sweet potatoes, and tomatoes, when expressed on the basis of lipid content, is surprisingly high. Thus many vegetable oils may be considerably richer in vitamin E content than wheat germ oil.

Emmerie and Engel ('43) reported essentially the same range of values for vegetables in Holland as that shown in table 1. Their samples of carrots and celery, however, were three to 4 times richer in total tocopherols. In addition, they found kale with 8.0 mg and parsley with 5.5 mg of total tocopherol per 100 gm to be among the richest vegetable sources of tocopherol.

Potatoes, both in Holland and in this country, are low in vitamin E potency. Emmerie and Engel's value of 0.1 mg and the value in table 1 of 0.06 mg of total tocopherols per 100 gm establish this widely used vegetable as a poor source of vitamin E.

Cereals and bakery products

The foods in this classification are generally considered the richest sources of vitamin E in man's diet. And it is evident from table 2 that the α-tocopherol content of whole grain cereals is relatively high, ranging from 0.84 mg per 100 gm for corn to 1.94 mg for oats. East Indian unpolished rice (Engel

and deVries, '46) and Dutch oatmeal, barley and rye (Emmerie and Engel, '43) are probably also in this range, judging from their high total tocopherol values.

TABLE 2

Tocopherol content of cereal and bakery products

FOOD	TOCOPHEROL CONTENT					
	mg/100 gm fresh material			% of total tocopherols	mg/gm extracted lipid	
	Total	$\gamma + \delta$	α	α	*Total*	α
Cereals:						
Cornmeal, yellow	1.70	0.86	0.84	49	1.7	0.8
Farina (breakfast food)	1.60	...	...	..	1.9	..
Oatmeal	2.10	0.16	1.94	92	0.3	0.2
Rice, brown	2.40	1.20	1.20	50	1.1	0.6
Rice, polished	0.57	0.22	0.35	61	1.2	0.7
Rice, polished, converted	0.23	...	...	..	0.8	..
Wheat, white flour (80% extraction)	1.20	...	...	..	0.9	..
Wheat, whole wheat flour	2.20	...	...	..	1.4	..
Wheat, spaghetti	1.20	...	...	..	1.3	..
Bakery products:						
Bread, white	0.23	...	...	..	0.2	..
Bread, whole wheat	1.30	...	...	..	0.8	..
Cake, light, unfrosted 1	9.65	7.06	2.59	27	0.5	0.1
Cake, light, unfrosted 2	8.42	4.03	4.39	52	0.6	0.3
Cookies, sugar 1	6.86	4.87	1.99	29	0.7	0.2
Cookies, sugar 2	9.97	4.96	5.01	50	0.7	0.3
Cookies, chocolate marshmallow	4.82	3.40	1.42	29	0.3	0.1
Cookies, tea, mixed	8.74	4.92	3.82	44	0.5	0.2
Crackers, soda	3.70	...	...	..	0.3	..
Doughnuts	2.52	1.74	0.78	31	0.1	0.04
Pie, apple 1	0.20	< 0.04	0.16	> 80	0.02	0.02
Pie, apple 2	0.29	0.10	0.19	66	0.04	0.03
Pie, apple 3	2.20	1.40	0.80	36	0.3	0.1
Pie, lemon cream	7.64	4.90	2.74	36	0.6	0.2
Pie shell, baked	0.87	0.38	0.49	56	0.2	0.1

The tocopherols of oatmeal are almost all α-tocopherol, whereas in most other cereals only about half the tocopherols are in the α- form. However, it was practically impossible to analyze wheat and wheat products for non-α-tocopherol content because of an anomalous color reaction which they give with diazotization reagents. Consequently only total tocopherol values are reported for wheat products, although from bioassay data we estimate that about 50 to 60% of the tocopherols present are in the α- form.

The very great loss of vitamin E due to the processing of cereals is illustrated in table 2 by the tocopherol values of rice and wheat. The consumer of polished rice obtains only about one-quarter of the vitamin E that he would from brown, unpolished rice. Engel ('46) reports a similar value, one-sixth, as a measure of the vitamin E retained in rice after polishing. Whole wheat flour loses about half its total tocopherol content when milled to white flour such as was used (80% extraction) in wartime in this country. If comparison were made between white and whole wheat breads using the tocopherol values reported in table 2, it would be concluded that whole wheat bread supplies a much more significant portion of the total daily intake of vitamin E. Assuming a daily consumption of 200 gm (8 slices) of bread, the use of white bread would supply only 0.46 mg of tocopherols, whereas whole wheat bread would furnish 2.60 mg.

Similar conditions exist in Holland. Both wheat and rye flour (80% extraction) possess only one-half to one-third the vitamin E of the whole grain from which they were milled. Furthermore, the bleaching of flour with NCl_3[2] and benzoyl peroxide destroys as much as 70% of the remaining vitamin E in wheat, rye, and barley flour (Engel, '42).

The vitamin E content of bakery products varies both because of variable losses due to the cooking process used and because of the type of ingredients used in the recipe. All of the cakes, cookies and pies listed in table 2 were commercial bakery products obtained from grocery stores and bakeries,

[2] Agene.

and no information was available concerning their composition. It is evident from the tocopherol content of these products, expressed on a fat basis (0.02 to 0.74 mg tocopherols/gm of fat), that lard, vegetable shortenings, and mixtures of animal and vegetable fats could have been and probably were used as ingredients. The destruction of vitamin E by the baking process is probably relatively slight although the extent of destruction cannot be ascertained from these data.

However, deep-fat cooking can destroy considerable vitamin E, as is exemplified by our results obtained with doughnuts. Samples of both doughnuts and cooking fat were obtained from a small commercial doughnut bakery where baking operations were typical of this industry. The shortening used as an ingredient in the doughnut recipe and to refill the cooking vat was a good quality, partially hydrogenated vegetable oil containing 92.8 mg of tocopherols per 100 gm. At the start of the day's cooking operation, this shortening was added to the fat remaining in the cooking vat from the previous day's baking. A sample of this mixture contained 19.4 mg of tocopherols per 100 gm. The fat from doughnuts in the first batch cooked showed a tocopherol concentration of only 12.1 mg/100 gm. At the end of the day a sample of fat from the cooking vat had a tocopherol content of 10.7 mg/100 gm, and the fat from doughnuts baked in the last batch contained 6.8 mg/100 gm. The extent of the loss of tocopherol due to deep-fat cooking is not merely from 19.4 mg %, the potency of the fat in the cooking vat, to 6 to 12 mg %, the fat in the cooked doughnuts, but must be measured from a value somewhere between 19.4 mg % and 92.8 mg %, the concentration of tocopherol in the shortening used in the doughnut dough.

Meats, fish, poultry and dairy products

It is evident from an examination of table 3 that foods in this category are relatively poor sources of vitamin E, with

the exception of eggs. Two eggs will supply 2.4 mg of tocopherol, about 60% of which is α-tocopherol.

The low tocopherol content of animal tissues may reflect loss during and after processing, since many fresh animal tissues obtained from animals on diets containing a minimum of vitamin E have shown a tocopherol content as high as 10 times the values in table 3. Stored tissues oxidize quickly and lose vitamin E, even though stored at low temperatures (Chipault, Lundberg and Burr, '45; Quaife and Dju, '49). The

TABLE 3

Tocopherol content of meats, fish, poultry and dairy products

FOODS	TOCOPHEROL CONTENT					
	mg/100 gm fresh material			% of total tocopherols	mg/gm extracted lipid	
	Total	γ + δ	α	α	*Total*	α
Meats:						
Bacon	0.53	< 0.09	0.44	> 83	0.01	0.01
Beef, steak	0.63	< 0.16	0.47	> 75	0.02	0.02
Beef, liver	1.40	0.00	1.40	100	0.3	0.3
Lamb, chops	0.77	< 0.15	0.62	> 81	0.03	0.02
Pork, chops	0.71	< 0.08	0.63	> 89	0.03	0.03
Fish:						
Haddock	0.39	< 0.04	0.35	> 90	0.7	0.6
Poultry:						
Chicken	0.25	< 0.04	0.21	> 84	0.05	0.04
Eggs, whole	2.00	0.84	1.16	58	0.2	0.1
Dairy products:						
Butter	2.40	...	...	..	0.03	..
Cheese, American	1.00	...	...	..	0.02	..
Ice cream, vanilla	0.30	...	...	..	0.05	..
Milk, whole fluid	0.12	...	...	..	0.03	..
Milk, evaporated	0.30	...	...	..	0.04	

low tocopherol values in this category are probably also due to the low dietary intake of the animals, since the vitamin E content of eggs, and of the other animal products too, has been shown to be dependent upon the level of vitamin E in the feed of the chicken, turkey, pig, and rabbit (Chipault, Lundberg and Burr, '45; Major and Watts, '48). When the level of vitamin E feeding is low, seasonally or by locality,

the food products from the animals will be correspondingly low, and vice versa.

The relatively high value for the vitamin E content of fat from haddock, as shown in table 3 and in Jansen and Kringstad's report ('42) that sardines have 4.5 mg tocopherol/100 gm (0.45 mg/gm of fat), suggests that fish may be a rich source of this vitamin. However, except for sardines and cod roe, other fish are reported (Jansen and Kringstad, '42; Kringstad and Folkvord, '49) to be lower in tocopherol content and, in general, in the same range as mammalian meats and poultry; e.g., salmon and mackerel 1.6 to 1.8 mg %, and herring 0.5 mg %.

Abderhalden ('47) reported an average concentration of 0.06 mg % of tocopherols in cows' milk, with a range of from 0.02 to 0.10 mg %. Milk in the Netherlands has been reported to contain 0.02 mg of tocopherol per 100 gm (Emmerie and Engel, '43). The value in table 3 of 0.12 mg per 100 gm is higher than those reported abroad and is explained on the basis of probable differences in the tocopherol level in the feed of the cows, although differences in the analytical procedures used may provide a partial explanation. In general, however, it may be concluded that cows' milk is a relatively poor source of vitamin E. One quart of milk would furnish only about 0.5 mg of tocopherol.

The processing of milk to make cheese, butter, or evaporated milk apparently does not destroy vitamin E. The tocopherol content of these products is similar to that of milk when expressed on a comparable basis (table 3) — 0.02 to 0.04 mg of tocopherols per gram of fat.

Oils and fats

An examination of table 4 reveals a remarkable difference in the vitamin E content of fats and oils of vegetable origin compared with those of animal origin. The former with few exceptions are rather good sources of vitamin E, while animal fats are uniformly quite poor sources. Several oils —

TABLE 4

Tocopherol content of oils and fats

FOOD	TOCOPHEROL CONTENT						
	mg/100 gm of sample			% of total	Literature values		
	Total	$\gamma + \delta$	α	α	*Total mg/100 gm*	α, % *of total*	*Reference*
Vegetable							
Coconut	8.3	4.7	3.6	43	..	..	
Coconut, hydrogenated	..	..	..	..	3	..	Quackenbush et al., '41
Corn, refined	87	80	7	8	250		Emmerie and Engel, '43
					90	10	Fisher, '45
					102	13	Quaife, '48
Cottonseed, refined	90	34	56	62	83	62	Hove and Hove, '44
					87	57	Quaife, '48
					91	71	Fisher, '45
Margarine,[1] clarified	54	26	28	52	8.9	..	Lundborg, '45
Olive	6.9	..	..	..	3	..	Emmerie and Engel, '43
Palm	56	26	30	54	44	..	Kofler, '43
					110	..	Emmerie and Engel, '43
Peanut, refined	22	11	11	50	26	..	Emmerie and Engel, '43
					36	63	Hove and Hove, '44
					11.2	..	Lundborg, '45
					48	50	Fisher, '45
					26.8	..	Tosić and Moore, '45
Rice bran	..	..	..	..	91	64	Fisher, '45
Soybean, refined	140	130	10	7	97	22	Fisher, '45
					23.4	..	Lundborg, '45
					125	..	Quackenbush et al., '41
					92	10	Hove and Hove, '44
					120	..	Emmerie and Engel, '43
					74	13	Quaife, '48
Wheat germ	..	..	..	..	150–250	..	Emmerie and Engel, '43
					274	70[2]	Hove and Hove, '44
					268	60[2]	Quaife, '48
					312	..	Tosić and Moore, '45
Animal							
Butter (80% fat)	2.4	..	..	..	2.5	..	Kofler, '43
					2.1–3.3	..	Emmerie and Engel, '43
Lard	2.7	< 0.4	2.3	> 85	1.0	..	Kofler, '43
					0.5	..	Chipault et al., '45
Shark liver	..	..	..	..	4–10	..	Robeson and Baxter, '43

[1] Margarines may be rich or poor sources of vitamin E, since they may have been manufactured from vegetable oils, animal fats, or mixtures of vegetable and animal fats.

[2] Remainder practically all β-tocopherol.

coconut, olive, and sesame — have relatively low concentrations of tocopherol, 3 to 18 mg per 100 gm, compared with other vegetable oils, but neverthless they are still superior to butter and lard with respect to vitamin E.

Soybean oil, as is characteristic of leguminous seed oils, and corn oil have only about 10% of their total tocopherols as α-tocopherol, whereas cottonseed, peanut, rice bran and

TABLE 5

Tocopherol content of miscellaneous foods

FOOD	TOCOPHEROL					
	mg/100 gm fresh material			% of total tocopherols	mg/gm of extracted fat	
	Total	γ + δ	α	α	*Total*	α
Chocolate, unsweetened	11.10	5.80	5.30	48	0.2	0.1
Cocoa (Emmerie and Engel, '43)	3.1	...	...	..	..	..
Peanuts	9.30	4.70	4.60	49	0.2	0.1
Potato chips	3.22	1.08	2.14	67	0.1	0.1
Pudding,[1] chocolate	0.69	0.0	0.69	100	0.3	0.3
Pudding,[1] vanilla	0.31	0.0	0.31	100	2.1	2.1
Yeast, dried brewers'[2]	0.0	0.0	0.0	...	..	..

[1] As purchased in dry powdered form.

[2] Yeast is devoid of vitamin E potency, as is evidenced by its use at a 10% level in the basal diet used in the standard vitamin E bioassay procedure. However, yeast fat does have reducing substances present which are measured as apparent tocopherols by the chemical method.

wheat germ oils have from 55 to 70% of their total vitamin E as α-tocopherol. The samples of margarine tested apparently were manufactured largely from vegetable oils, judging by the relatively high concentration of total tocopherols. Also, because of the low value found for the ratio of α- to total tocopherol, these oils were probably soy or corn oils, or both.

Miscellaneous

A knowledge of the concentration of tocopherol in the miscellaneous list of foods in table 5 is helpful in making dietary analyses for vitamin E. Chocolate is the only food on the list especially rich in this vitamin. Chocolate as candy bars, which sometimes make up as much as 10% of the caloric content of diets (McCay et al., '45), would supply an appreciable and probably significant quantity of α-tocopherol to the consumer.

The high value for peanuts (table 5), 9.3 mg of tocopherols per 100 gm, of which about 50% is α-tocopherol, establishes it as an excellent food source of vitamin E and arouses interest in the vitamin E content of other nuts. Lundborg ('45) found 11.2 mg of total tocopherol per 100 gm of peanuts, and Emmerie and Engel ('43) report high tocopherol values for beechnut and palm oils, indicating that the original nuts were probably rich sources of vitamin E.

DISCUSSION

With values available for the vitamin E content of foods, it is a temptation to evaluate various standard diets to obtain an estimate of the human daily intake of this vitamin, or more significantly, of α-tocopherol. Hickman and Harris ('46) have already speculated that the daily dietary intake for individuals may range between 1.5 and 44 mg of *d*, α-tocopherol (3 to 88 mg of mixed natural tocopherols), depending upon socio-economic status. In Holland, Engel ('46) calculated that an average of 15.7 mg (a range of from 10.7 to 23.9 mg) of total tocopherols were consumed daily. This amounts to about 8 mg of *d*, α-tocopherol, assuming that approximately 50% of the natural mixed tocopherols in foods are in the α- form. Quaife, Swanson, Dju and Harris ('49) reported an estimated daily intake of α-tocopherol of from 4 to 9 mg for individuals on reducing, low-sodium, or diabetic-type diets, or those receiving only the basic foods recommended by the National Research Council. For in-

dividuals on a "fattening" diet, an intake of from 10 to 27 mg of α-tocopherol was estimated.

A slightly different approach to an estimation of per capita food consumption may be made from data compiled by the United States Department of Agriculture ('44). The sum total of all of the foods sold commercially in the United States, divided by the population, gives values for each of

TABLE 6

Vitamin E content of the average daily per capita food consumption in the U. S. in 1948 (U.S.D.A., '49)

COMMODITY	AVERAGE CONSUMPTION	VITAMIN E INTAKE	
		Total tocopherol	α-Tocopherol
	gm/day	*mg/day*	*mg/day*
Fats and oils, incl. butter	80.8	14.42	7.87
Grain products	212.5	2.85	1.78
Meat, poultry and fish	196.4	1.17	0.99
Potatoes and sweet potatoes	142.9	0.87	0.84
Eggs	58.4	1.16	0.68
Dairy products, excl. butter	535.6	0.58	0.58
Green leafy and yellow vegetables	141.7	1.15	0.50
Dried peas, beans and nuts	19.9	1.23	0.43
Citrus fruits and tomatoes	130.5	0.38	0.35
Other vegetables and fruits	290.8	0.17	0.12
Coffee, tea and cocoa	23.6	0.13	0.06
Sugar and syrups	131.7	0.00	0.00
Totals	1,964.8	24.12	14.20 (59% of total tocopherols)

the foods which, if used as consumption values, must be accepted as maximum. This is true because much of the foodstuff sold is not actually consumed but is lost during transportation or display in retail stores, or wasted during preparation and cooking. Nevertheless, the average individual food consumption calculated in this way would furnish about 19 mg (17.5 I. U.) of α-tocopherol daily. Thus, this value would seem to be the maximum obtainable by the

average American. More recent government values for average per capita food consumption (U. S. Dept. of Agriculture, '49) indicate that 14 mg (13 I.U.) of α-tocopherol is a more likely value for the mean daily intake of vitamin E, since it is based on directly measured per capita food consumption as shown in table 6.

SUMMARY AND CONCLUSIONS

Commonly used foods, typified by those available in Rochester, N. Y., have been chemically analyzed for their total tocopherol and α-tocopherol content.

On the basis of the results obtained, the average per capita consumption of vitamin E in the United States has been estimated as 14 mg of *d*, α-tocopherol (13 I.U.) daily.

The richest dietary sources of vitamin E are certain of the vegetable oils. Cereal products and eggs are next in order of nutritional importance. Vegetables supply very little of the daily intake of vitamin E, both because of their low concentration of total tocopherols and because in most instances a large portion of the tocopherols present are the relatively inactive, non-α-tocopherols. Animal products, similarly, are rather poor sources of vitamin E because of their quite low and variable concentration of total tocopherols, practically all of which, however, is α-tocopherol.

The tocopherol concentration in animal products is probably directly proportional to the level of vitamin E in the diet of the animals from which they come and, inversely, to the severity of processing and length of storage to which they are subjected.

The value of cereal products as a source of vitamin E certainly depends upon the extent and severity of the milling process which they undergo prior to consumption.

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THE IMPORTANCE OF THE DIETARY LEVEL OF FATS ON THEIR NUTRITIONAL EVALUATION[1]

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In most of the work so far carried out on the comparative value of animal and vegetable fats, a single arbitrary dietary level has been used. It seems likely that the optimum dietary level for one fat may be different from that of a second. If such were the case, it would not be satisfactory to compare two fats at any one dietary level.

Hoagland and Snider ('40) compared the nutritive properties of 8 animal and vegetable fats at 5, 30 and 55% levels in isocaloric diets. The growth of the rats was improved when the levels of hydrogenated lard, leaf lard, neutral lard, oleo and peanut oil were increased. Increased levels of cottonseed oil resulted in reduced growth, while the best growth on the refined lard and the hydrogenated cottonseed oil diets was obtained when these constituents made up 30% of the rations. When steam-rendered lard and hydrogenated cotton-

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Some of the early work reported in this paper was carried out in cooperation with Dr. H. Nath of India.

seed oil were fed at 5, 15, 30 and 54% levels, maximum growth resulted when these two fats constituted 30% of the diets (Hoagland and Snider, '41).

Deuel and co-workers ('47) fed increasing levels (5 to 50%) of cottonseed oil and margarine fat in isocaloric diets,

TABLE 1

Composition of the diets

BASAL MIXTURE				VITAMIN SUPPLEMENTS ADDED TO DIET 2 [3]	
Component	Diet 1 10% fat	Diet 2 28% fat	Diet 3 35% fat		mg
	gm	gm	gm	Thiamine	0.20
Fat	10	28	35	Riboflavin	0.30
Sucrose	66	43.87	35.22	Pyridoxine	0.30
Casein [1]	20	24.13	25.78	Ca pantothenate	1.50
Salts IV [2]	3.32	4	4.21	Choline	150.00
Calories per 96 gm of energy yielding food	434	524	558	Carotene [4]	0.56
Grams protein per calorie from the above diets	0.04615	0.04615	0.04615	α-Tocopherol	2.24
Ratio for feeding isocaloric amounts	1.274	1.062	1	Calciferol [5]	0.014
				2-Me-1,4-naphthoquinone	0.21

[1] Extracted for three two-hour periods with boiling alcohol.

[2] Hegsted et al. ('41).

[3] The vitamin supplements added to diet 1 were 0.828 of the amounts listed, in order to supply equal amounts of the vitamins per calorie; for the same reason 1.066 times the amounts of vitamin supplements listed were added to diet 3.

[4] Ninety per cent β-carotene and 10% α-carotene.

[5] Crystalline irradiated ergosterol.

and observed optimum growth in male rats when the ration contained 30 to 40% of margarine or 20% of cottonseed oil.

Forbes et al. ('46) pair-fed isocaloric diets containing 2, 5, 10 and 30% of fat (lard) to growing rats and found increasing weight gains in the order of the increasing fat content of the diet, while Boutwell et al. ('43) fed 25, 30 and 35% of corn oil with skim milk powder diets and observed reduction in weight gains with the increased level of the oil.

The experiments reported in this paper were undertaken in order to study the effect of changing the level of different fats in the ration of growing rats and to determine the more nearly optimum range of each fat in the diet of the albino rat.

EXPERIMENTAL AND RESULTS

Twenty-one-day-old rats of the Sprague-Dawley strain weighing 40 to 45 gm were used in all the experiments. They were housed in individual metal cages with raised screen

TABLE 2

Results of experiment 1

(Average[1] gain in grams per 6 weeks of paired feeding, and the amounts of body fat in rats fed diets containing various levels of butterfat and corn oil. Eight rats per group)

Diet no.	1	2	3
Fat level	10%	28%	35%
Butterfat			
weight gains	169.4 ± 2.05	173.8 ± 1.90	179.8 ± 3.30
gm fat/rat	27.9	29.1	35.1
% body fat	13.2	13.5	15.8
Corn oil			
weight gains	174.5 ± 2.26	182.1 ± 1.38	176.7 ± 3.73
gm fat/rat	24.0	31.8	34.4
% body fat	11.1	14.3	15.7

[1] Including the standard error of the mean as calculated from the formula

$$s_{\bar{x}} = \sqrt{\frac{\Sigma (X - \bar{x})^2}{n (n - 1)}}.$$

bottoms, and weighed once weekly. Feed and water were provided daily ad libitum unless otherwise indicated. Isocaloric diets (table 1) were used in the experiments described. These diets were so compounded that the ratio of calories to proteins, salts and vitamins was constant in spite of the increasing amounts of fat. Thus, the intake of protein, minerals and vitamins would be proportional to the caloric intake in the ad libitum feeding and equal in all diets in the paired

feeding regimen. The diets were mixed approximately once weekly and were stored in a refrigerator.

All growth data presented have been analyzed statistically. Fisher's "*t*" test was carried out to determine significance according to Snedecor ('46). A probability of less than 0.05 has been considered significant and one of less than 0.01 highly significant.

Four experiments are reported. In the first experiment the rats were pair-fed, i.e., they were restricted in food consumption to the least amount consumed by any single rat. The plan of the experiment, as well as the results obtained, are represented in table 2. It was observed that during the entire experiment the group on the 35% corn oil diet determined the consumption of all the other groups. It will be noted that there was a significant increase in gain with the increase of butterfat in the diets (10% to 35%, $P = 0.018$). On the corn oil diets the highest gains were made by the animals on the 28% diet; this growth was also significantly higher than that on the corresponding diet containing 28% butterfat ($P = < 0.01$).

Feces were collected throughout the entire experiment, kept under refrigeration and analyzed for neutral and for acid-hydrolyzable lipids. The values obtained for the fat content of the feces were corrected for metabolic fat on the basis of caloric intake and body weight. The results of this and of the second experiment are summarized in table 4, which also includes values for the utilization of the diets. At the end of the experiment, after the rats were killed and the gastro-intestinal contents washed out, the fat content of their bodies was determined. These values are given in table 2.

The second experiment was similar to the first, except that the groups consisted of 6 rats each and were fed ad libitum, and was conducted concurrently. The outline and results of this experiment are given in table 3. As in experiment 1, the growth rates on the butterfat diets increased significantly with increased levels of fat (10% to 35%, $P = < 0.01$). In the case of corn oil, the lower levels gave sig-

TABLE 3

Results of experiments 2, 3 and 4 (Average[1] gain in grams per 6 weeks)

Diet no.			1	2	3
Fat level			10%	28%	35%
EXP. NO.		RATS/ GROUP			
2	Butterfat	6	185.5 ± 7.03	205.3 ± 9.27	220.2 ± 3.41
	Food consumed in calories/6 wks./rat[2]		2219	2358	2411
	Corn oil	6	215.2 ± 7.36	211.5 ± 6.51	186.7 ± 4.80
	Food consumed in calories/6 wks./rat[2]		2354	2410	2062
3	Butterfat	6	187.3 ± 7.18	191.8 ± 5.59	206.8 ± 9.09
	Butterfat + 1 drop ethyl linoleate/day	6	179.3 ± 5.41	..	..
	Corn oil	6	..	208.8 ± 7.33	194.5 ± 3.46
	Corn oil + liver extract	6	..	198.3 ± 6.36	189.0 ± 7.59
	Soybean oil	6	199.7 ± 8.37	200.5 ± 6.15	190.7 ± 7.35
	Coconut oil	6	174.5 ± 4.80	178.2 ± 7.71	178.0 ± 8.85
4	Butterfat	8	188.9 ± 7.32	213.5 ± 2.30	231.5 ± 6.56
	Butterfat + 1 drop ethyl linoleate/day	8	199.1 ± 5.39	..	..
	Butterfat + 1 drop corn oil/day	8	192.7 ± 5.36	..	..
	Corn oil	8	204.1 ± 6.28	201.2 ± 9.71	205.2 ± 7.12
	Soybean oil	8	204.8 ± 7.75	203.9 ± 6.95	201.9 ± 6.16
	Coconut oil	8	190.6 ± 4.37	191.2 ± 6.88	197.2 ± 4.83
Average of groups repeated in exps. 2, 3 and 4	Butterfat	20	187.4 ± 4.21	204.5 ± 3.34	220.3 ± 4.34
	Corn oil	20(14)[3]	208.9 ± 4.76	206.6 ± 4.89	196.4 ± 3.38
	Butterfat + ethyl linoleate	14	189.2 ± 3.86	..	..
	Soybean oil	14	202.3 ± 5.70	202.2 ± 4.78	196.3 ± 4.72
	Coconut oil	14	182.5 ± 3.24	184.7 ± 5.14	187.6 ± 4.66

[1] Including the standard error of the mean as calculated from the formula

$$s_{\bar{x}} = \sqrt{\frac{\Sigma (X - \bar{x})^2}{n (n - 1)}}.$$

[2] Calories consumed represent also the proportional consumption of the other constituents of the isocaloric diets.

[3] The 10% corn oil average includes only 14 rats.

nificantly higher growth rates (10% to 35%, P =<0.01). Food consumption records were kept throughout the experiment and the calculated efficiency of utilization is given at the bottom of table 4. There was improved utilization with increased butterfat in the diet in both the ad libitum and the

TABLE 4

Summary of results with respect to digestibility and utilization of the diets as obtained from experiments 1 (paired feeding) and 2 (ad libitum)

		NO. OF RATS	BUTTERFAT			CORN OIL		
			10%	28%	35%	10%	28%	35%
% fatty acid and neutral fat in feces	ad lib.	6	9.71	8.13	8.44	18.6	25.1	27.5
	pair-fed	8	10.2	8.24	7.65	16.0	29.4	24.9
% acid hydrolyzed fat (soaps) in feces [1]	ad lib.	6	34.8	46.7	51.6	13.7	18.1	21.5
	pair-fed	8	40.4	47.3	57.8	14.3	14.2	15.6
% total lipids in feces [1]	ad lib.	6	44.5	54.8	60.0	32.3	43.2	49.0
	pair-fed	8	50.6	55.5	65.5	30.3	43.6	40.5
gm lipids excreted/ rat per 6 weeks [2]	ad lib.	6	4.3	7.9	10.1	1.7	5.0	4.8
	pair-fed	8	4.2	6.7	8.1	1.5	3.6	3.3
Coefficient of digestibility	ad lib.	6	91.6	93.7	93.3	96.9	96.1	96.3
	pair-fed	8	90.8	93.7	93.5	96.7	96.6	97.3
Efficiency of utilization (gm gain/100 calories absorbed)	ad lib.	6	8.49	8.98	9.52	9.14	8.91	9.26
	pair-fed	8	8.72	9.06	9.42	8.87	9.35	9.07

[1] Includes metabolic fat.

[2] Metabolic fat subtracted.

paired feeding experiments. No correlation was evident between the level of fat and the growth efficiency of the diets when corn oil was used.

In the third experiment an attempt was made to determine the causes for reduced growth on the diets containing low levels of butterfat and those containing high levels of corn oil. Insufficient amounts of essential fatty acids were suspected to be the reason in the case of the 10% butterfat diet, while previous experiments in which non-isocaloric diets

were used (Barki, '49) indicated that a commercial liver extract [2] administered orally seemed to counteract the growth depressing effect of the high corn oil levels. Therefore, the rats in the group receiving the 10% butterfat diet were supplemented with one drop (approximately 50 mg) of ethyl linoleate per rat per day, and two groups receiving the 28 and 35% corn oil diets were given an intraperitoneal injection of 0.1 unit of the commercial liver extract per rat per day. In this experiment studies were also made of the effect of increasing the levels of soybean oil and coconut oil in similar isocaloric diets. The weight gains for the individual groups are listed in table 3 under experiment 3.

Although the previously observed effect of changing the level of butterfat and corn oil in the diets is again indicated, it in no case is statistically significant. It will be noted that the ethyl linoleate did not produce a change in the rate of growth on the 10% butterfat diet. In contrast with previous experiments (Barki, '49) in which the liver extract was administered orally, no improved growth was observed when the rats on diets containing 28 and 35% of corn oil were given the liver extract by intraperitoneal injection. The change in the level of soybean or coconut oil in the diet did not alter the growth rate significantly, although in general the gains on the coconut oil diets were low.

The 4th experiment was set up to check previous observations systematically. The outline of the experiment and its results are presented in table 3. The increased rate of growth due to the higher levels of butterfat is obvious and significant (10% to 35%, $P=<0.01$). The addition of the essential fatty acids, pure or in corn oil, produced no significant effect. In this experiment the increased level of corn oil in the diets did not result in the growth depressing effect observed in the previous experiments. The results with the soybean and coconut oil diets were very similar to those obtained in the previous experiment, although in this ex-

[2] Lilly, Reticulogen, 20 U.S.P. units per milliliter.

periment somewhat improved growth was achieved in general on the three coconut oil rations. This improvement approaches statistical significance in the case of the 35% diet ($P = 0.081$).

The groups in the ad libitum experiments which occurred more than once are averaged in proportion to the number of rats included. These means with their standard errors are shown at the bottom of table 3.

DISCUSSION

The data presented demonstrate repeatedly that the rate of growth of rats was affected by changing the level of different fats in the diets (tables 2 and 3). Thus, without exception, raising the level of butterfat from 10 to 35% resulted in improved growth. On the other hand, improvement in growth was secured when the level of corn oil was decreased from 35 to 10% (table 3). Our results with varying levels of these two fats agree with the findings of Boutwell et al. ('43). In the cases of soybean oil and coconut oil, the influence of changing the fat level on the growth rate was not significant. However, soybean oil appeared to follow the pattern demonstrated by corn oil, while coconut oil followed that set by butterfat, although the rate of growth on the coconut oil diets was generally lower than on the butterfat diets (table 3). Our observations in general agree with those of Hoagland and Snider ('40, '41) who also found that different fats might have different optimum levels for the growth of rats.

The improved growth and efficiency obtained in the paired feeding trial with the increased butterfat level in the diets are in agreement with the conclusions of Forbes et al. ('46). However, these workers generalized from a study of the effects of using varying levels of a single fat (lard) and presumably interpret their results as representing the behavior of fats in general. Our findings do not support such a generalization; while we found that butterfat certainly behaved in this manner, i.e., improved growth and efficiency with higher dietary levels, corn oil (in paired feeding experi-

ments) followed this trend only when it was increased from 10 to 28%, and an opposite trend when it was increased from 28 to 35%. It should again be pointed out that in experiments 2 and 3 the ad libitum feeding of corn oil resulted in a decrease in growth with every increase between 10 and 35%.

On the basis of our present knowledge it is difficult to explain with any degree of certainty the slower growth obtained with the 10% butterfat and 35% corn oil diets. Nath et al. ('48) analyzed the cecal flora of rats on similar diets and found the number of coliform organisms to have been decreased in the ceca of most of the rats fed sucrose diets containing a high level of corn oil. It is generally believed that this group of microorganisms is responsible to a great extent for the intestinal synthesis of vitamins. On the basis of the above study it would seem that high levels of corn oil exert their effect directly on the microflora and indirectly on the animal. Although we used a diet containing 35% corn oil in order to observe this effect, Boutwell et al. ('45) came to a similar conclusion when they used 28% corn oil diets.

In the study mentioned above, Nath et al. also found that the supplementation of a high corn oil-sucrose diet with a commercial liver extract [3] tended to counteract the inhibitory action of the corn oil on the growth of certain microorganisms. The observations quoted from previous experiments (Barki, '49) in which the liver extract was administered orally and found to counteract the growth depressing effect of high corn oil levels are in line with the findings of Nath et al. In fact, the failure to effect a growth improvement of the intraperitoneal injections of liver extract in experiment 3 of the present study may also support the suggestion of an effect and a countereffect on the intestinal flora.

We are unable to explain the observation that at the 10% level growth on the butterfat diet was significantly inferior to that on the corn oil or soybean oil rations. Attempts to improve it by the addition of essential fatty acids failed.

[3] Reticulogen.

In the light of the above findings it would seem unjustified to compare the nutritive value of different fats on the basis of weight gains, using any one particular level of dietary fat.

SUMMARY

Butterfat, corn oil, soybean oil and coconut oil were fed to rats at three different levels in the diet, 10, 28 and 35%. The rats gained the most weight on the 35% butterfat diet and the 10% corn oil diet. Slower growth was observed on the diets containing higher levels of corn oil and lower levels of butterfat. Changes in the levels of coconut oil or soybean oil in the diet did not result in significant changes in growth, although in general the gains on the coconut oil diets were low.

These observations suggest that since different fats produce optimum growth at different levels of intake, a comparative evaluation of their nutritive merits based on only one dietary level may be misleading.

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THE PREVENTION OF CARIES IN THE SYRIAN HAMSTER WITH SODIUM OXALATE [1]

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The carious lesions that develop in the teeth of experimental animals maintained on high sugar diets are generally believed to result from the activity of acidogenic bacteria present in the oral cavity. Lactic acid produced from carbohydrate by the glycolytic enzymes of the acidogenic bacteria is held to be responsible for the enamel destruction.

Gortner, McCay, Restarski and Schlack ('46) demonstrated that the presence of soluble oxalates in the food or drinking water of the rat effectively protected the molars of this animal from the decalcifying action of ingested phosphoric or citric acids. This observation suggests that oxalates might also protect the teeth against the action of acids arising as a result of bacterial action, and prevent the development of carious lesions.

The experiments reported below were designed to study the effect of oxalate on caries' incidence in the Syrian hamster subsisting on a high-sugar, cariogenic diet. The extent to which the oxalate ion was incorporated in or adsorbed upon the teeth of the experimental animals was determined by chemical analysis.

The hamster was chosen as the experimental animal because of the ease with which caries can be produced in this

[1] This paper was prepared from the thesis presented by Robert M. Twedt to the Graduate School of the University of Colorado for the M.S. degree, June 1949.

animal on a high carbohydrate diet (Keyes, '46). Keyes and Dale ('44) have suggested that the hamster can be used profitably in caries research because its teeth, unlike the rat's, are completely covered with enamel and have a morphology similar to that of human teeth. The distribution of carious lesions in the teeth of the animals and the differences in caries susceptibility between male and female hamsters have been recorded.

METHODS

Five litter-mate groups of hamsters were used in these experiments. Beginning at 30 days of age, each group was fed the cariogenic, high-sugar diet described by Keyes ('46). This consisted of whole wheat flour 20%, corn starch 25%, confectioners sugar 20%, powdered whole milk 30%, and ground, dehydrated alfalfa 5%. The first group, I, was fed this diet for 61 days; the second group, II, for 125 days; and the third group, III, for 157 days. In the diet of the two remaining groups, IV and V, sodium oxalate was added to a level of 0.5%, replacing an equal portion of corn starch. Group IV received this diet for 128 days and group V for 142 days.

At the end of the experimental period for each group, the animals were sacrificed and the maxilla and mandible removed and each halved. The fresh tissue was scraped from each jaw, and each half was allowed to dry in a test tube. The caries in each molar were recorded by inspection with the aid of a dissecting microscope and the caries index scored according to the method of Keyes ('44). The caries indices were compared: (1) between cariogenic and oxalate groups in the same time period; (2) between sexes of the same group; and (3) between sexes in cariogenic and oxalate groups.

After the caries had been recorded, the crowns from each half jaw were severed at the gingival line and the crowns from each animal pooled. Oxalate determinations were made upon the pooled tooth substance of each animal according

to the method of Calkins ('43). In this method oxalic acid is reduced to glycolic acid and the color developed by the action of glycolic acid and 2, 7-dihydroxynaphthalene is compared with an appropriate standard.

The pooled crowns of each animal were dissolved in 4 ml of concentrated H_2SO_4. Three aliquot portions of 0.1 or 0.4 ml were removed, and each diluted to 4 ml with concentrated H_2SO_4. Reduction was carried out with 5 mg of powdered magnesium, the solution being agitated nearly continuously for one hour. Then 2 ml of a 0.01% solution of 2, 7-dihydroxynaphthalene were added to each aliquot tube. Color was developed by heating in a boiling water bath for 20 minutes. The color was read in a Klett-Summerson electrophotometer against a control tube made in an identical manner, leaving out the color reagent and adding 2 ml of concentrated H_2SO_4 in its place. A standard curve, against which the color was compared, was made by reducing 0.2 ml of known oxalic acid solutions with 5 mg magnesium, diluting to 4 ml with concentrated sulfuric acid, and proceeding as before with the color reagent.

The method proved to be adequate for the determination of the small quantities of oxalic acid present in the tooth solutions. When known amounts of oxalate were added to solutions of the teeth, an average of 90% of the added oxalate could be recovered with this method. The average error of duplicate determinations in the analysis of tooth substance for oxalate was 1.8%. Calkins ('43) found that none of the more common organic acids interfered in the determination, including organic acids such as acetic, succinic and citric, which might be present in a tissue solution. However, in the analysis of the teeth of the hamsters of groups II and III, some color developed. It is improbable that these animals had ingested any appreciable amount of oxalate from the diets, unsupplemented with sodium oxalate. While expressed as oxalic acid, these values probably represent the basic amount of some interfering substance present in solutions of tooth substance.

RESULTS

In agreement with the findings of Keyes ('46), some caries were observed in the teeth of the hamsters after 61 days on the cariogenic diet. The average caries indices were not large, however. For this reason the observations on caries and on the effect of dietary oxalate were largely confined to groups

TABLE 1

Average caries indices and average oxalate content of the teeth of hamsters on cariogenic diets, supplemented and unsupplemented with sodium oxalate

GROUP	SEX	NUMBER OF ANIMALS	TIME ON DIET	SODIUM OXALATE IN DIET	CARIES INDEX	OXALIC ACID PER MILLIGRAM OF TOOTH SUBSTANCE
			days	%		*µg*
I	M	4	61	0	3.0	
	F	2			4.4	
II	M	3	125	0	18.2	3.3
	F	6			6.2	
III	M	3	157	0	72.7	1.6
	F	1			30.0	
IV	M	1	128	0.5	5.2	11.8
	F	4			4.2	
V	M	3	142	0.5	4.4	10.2
	F	4			6.7	

II to V. The hamsters in these groups had been on the high carbohydrate diet for 125 days or longer, and pronounced carious activity had developed in the teeth of animals receiving no sodium oxalate.

The average caries indices for the animals in each group and the average oxalate content of the teeth are summarized in table 1. It is evident from the data that rampant caries may be expected in the hamster after 100 days on the ex-

perimental diet, particularly in the male animals, whose teeth seem to be more susceptible to carious lesions than those of the females. Low caries indices were noted in both groups of animals receiving the cariogenic diet for 128 and 142 days when it was supplemented with sodium oxalate. It is evident from the analysis of the teeth of the animals of these two groups that an appreciable amount of oxalate had been taken up by the teeth.

TABLE 2

Average values for molars affected, caries number per jaw, areas affected per jaw, and caries indices for group II, fed a cariogenic diet for 125 days

JAW	SEX	MOLARS AFFECTED	CARIES NUMBER	AREAS AFFECTED	CARIES INDICES
Left maxilla	Male	2.3	4.3	2.7	6.2
Left maxilla	Female	1.7	2.3	1.2	2.4
Right maxilla	Male	3.0	4.7	2.4	5.5
Right maxilla	Female	2.2	2.5	1.6	3.4
Left mandible	Male	1.3	2.3	1.3	2.9
Left mandible	Female	0.5	0.7	0.2	0.4
Right mandible	Male	2.0	4.0	2.1	3.6
Right mandible	Female	0.2	0.2	0.1	0.1

The average caries indices for all jaws of the animals in group II which were fed the cariogenic diet for 125 days exhibit a marked difference between males and females (table 2). It will also be noted in this table, which records the distribution of caries in the jaws of the animals, that the maxillary molars of both sexes had higher caries indices than the mandibular molars. This distribution of caries in the hamster is in agreement with that similarly observed by Keyes ('46). The animals in group III showed a similar sex difference in caries incidence and in distribution of lesions between maxillas and mandibles as was found in group II. The average caries indices of the left and right maxillas of the

males was 20.8 and 23.3, respectively, while the average caries indices of the left and right mandible were 15.4 and 13.1, respectively. The scoring of the females showed an average index of 7.2 for the left maxilla and 6.5 for the right maxilla. The indices for the left and right mandibles were 7.5 and 8.8.

TABLE 3

Oxalate content of teeth of hamsters maintained on a cariogenic diet, supplemented and unsupplemented with sodium oxalate

DIETARY OXALATE = 0				DIETARY OXALATE = 0.5%			
Group	Animal	Sex	Oxalic acid per milligram of tooth substance	Group	Animal	Sex	Oxalic acid per milligram of tooth substance
			μg				μg
II	1	M	1.4	IV	1	M	8.9
	2	M	0.6		2	F	8.1
	3	M	4.2		3	F	15.0
	4	F	5.2		4	F	9.7
	5	F	1.2		5	F	17.5
	6	F	3.4	V	1	M	9.1
	7	F	5.3		2	M	13.2
	8	F	4.0		3	M	14.8
	9	F	4.2		4	F	8.9
III	1	M	2.5		5	F	6.9
	2	M	2.7		6	F	11.6
	3	M	0.0		7	F	6.9
	4	F	1.3				

The most striking feature of the jaws of groups IV and V is the low caries index after 128 and 142 days on the cariogenic diet supplemented with sodium oxalate. It is interesting to observe the similarity of the average caries index of these two groups to the index of group I (table 1). The minimum caries indices observed in group I after 61 days on the cariogenic diet were not increased but were preserved by the oxalate-containing diet of groups IV and V. This is in contrast to the large increase in caries indices observed in groups II and III. As would be expected from the low indices, the sex differences and the maxillary-mandibular differences were not pronounced in groups IV and V.

No evidence of gingival caries was found during scoring of the teeth. All caries recorded were located in the occlusal and interproximal sulci and fossae. Keyes ('46) has described several different kinds of lesions obtained in hamster teeth and concluded that gingival caries were characteristic of dental caries in the hamster. It is possible that hereditary factors which may control the areas most susceptible to decay are responsible for the differences between the teeth of Keyes' animals and those of the hamsters used in the present experiments.

The results of the oxalate analysis of the pooled crowns of each animal are given in table 3.

DISCUSSION

It is evident from the results of these experiments that the incorporation of sodium oxalate into a high-carbohydrate, cariogenic diet at a level of 0.5% effectively protects the Syrian hamster from dental caries. The minimum concentration of ingested oxalate that is necessary to afford protection has not been determined. Gortner et al. ('46) found that a level of 0.1% oxalic acid afforded complete protection against etching of rats' teeth by organic juice. Howell, Schlack, Taylor and Berzinskas ('48) observed no protection against experimental caries in the cotton rat afforded by potassium oxalate present in the rats' drinking water in concentrations of 0.2% or less.

The teeth of the hamsters with low caries indices after the ingestion of sodium oxalate contained appreciable amounts of oxalic acid, in contrast to the teeth of the animals receiving no oxalate (table 3). It seems reasonable to conclude that the protection against caries observed in the animals receiving oxalate is associated with the presence of this ion in the tooth substance. It is possible, as Gortner et al. ('46) found, that ingested oxalate might have been precipitated upon the teeth by combining with calcium ions in the saliva. Thus, a less soluble protective layer would stand between the enamel of the teeth and the acid produced in the mouth.

In the present experiments no precipitate was noticed upon any of the teeth of the animals fed sodium oxalate, but some of the teeth revealed crystals impacted in fossae and in interproximal spaces. It would seem probable that if the oxalate found by analysis in the teeth of the oxalate-fed animals were precipitated on the surface, more consistent tartar would have been observed on the teeth of all the animals in groups III and IV. Buonocore and Bibby ('45) have shown that the presence of the oxalate ion reduces the solubility of pulverized enamel in acids. It is possible that the oxalate ion was incorporated into the chemical structure of the teeth to form an oxal-apatite that would require a lower decalcifying pH than the acidogenic organisms produce in the oral cavity. A similar explanation for the protective effect of fluoride against dental caries has been advanced by many workers. A number of years ago Volker ('39) showed that the solubility of enamel or dentine in acid was decreased after exposure of these substances to solutions of sodium fluoride. On the other hand, fluoride inhibits the production of lactic acid from sugar by *Lactobacillus acidophilus* (Bibby and Van Kesteren, '40), and the protective action of fluorides may result from its role as an enzyme inhibitor. In the case of oxalate, until it has been shown that this ion in low concentrations is an inhibitor of acidogenic organisms, its protective action against carious lesions must be regarded as being localized in the enamel or on the tooth surface.

SUMMARY

Young Syrian hamsters, placed on the high-carbohydrate diet of Keyes, developed carious lesions in the occlusal and interproximal sulci and fossae of their teeth. A greater degree of caries activity was observed in the teeth of male animals than in the teeth of females.

The incorporation of sodium oxalate into the cariogenic diet at a level of 0.5% protected the hamster from caries. Chemical analysis of the tooth substance of the animals protected from caries by ingested oxalate revealed that sig-

nificant amounts of the oxalate had been incorporated into the teeth. On the basis of these findings, the mechanism of the protective action of oxalate has been discussed.

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CRYSTALLINE VITAMIN B_{12} IN THE NUTRITION OF THE BABY PIG

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THREE FIGURES

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In previous papers we have reported the development of of a synthetic milk ration which is deficient in the unknown growth factors contained in purified casein. It was found that this factor could be supplied to the baby pig by antipernicious anemia liver extract (Johnson and Neumann, '48; Neumann, Krider and Johnson, '48).

The purpose of the present experiment was to study the comparative effect of crystalline vitamin B_{12} and antipernicious anemia liver extract on growth and blood cell formation in baby pigs fed the synthetic milk ration. A preliminary report of this work has been published (Johnson and Neumann, '49).

EXPERIMENTAL

Ten 48-hour-old Duroc pigs and three cross-bred pigs (Duroc $\times$ Poland China) from the University farm were used in the experiment. The technique of feeding and care of the animals was similar to that reported by Johnson, James and Krider ('48). The pigs were fed ad libitum the basal alpha-protein [1] "synthetic milk" ration, the composition of

[1] Alpha-protein, an isolated soybean protein, obtainable from the Glidden Company, Chicago, Illinois.

TABLE 1

Composition of alpha-protein "synthetic milk"

INGREDIENT [1]	AMOUNT	VITAMINS ADDED PER LITER			
	%		*mg*		
Alpha-protein [2]	29.4	Thiamine [5]	0.65	Biotin	0.01 mg
DL-methionine [3]	0.6	Riboflavin	1.30	Pteroylglutamic acid	0.052 mg
Glucose (cerelose)	30.9	Pyridoxine	1.30	Ascorbic acid	16.00 mg
Lard	30.8	Ca pantothenate	7.80	α-Tocopherol acetate	1.00 mg
Mineral salts [4]	8.3	Inositol	26.0	2-Methyl-1,4-naphthoquinone	0.26 mg
		Nicotinic acid	2.6	Vitamin A	2,000 I.U.
		p-Aminobenzoic acid	2.6	Vitamin D_2	200 I.U.
		Choline chloride	130.0		

[1] These materials were homogenized into a "synthetic milk" containing 13% solids and 4% fat.

[2] Alpha-protein was obtained from the Glidden Company, Chicago, Illinois.

[3] DL-methionine and crystalline vitamin B_{12} were supplied by Merck and Company, Inc., Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

[4] Salts used by Johnson et al. ('48), except that Ca lactate replaced $CaCO_3$ in equimolar amount.

[5] Thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, biotin, nicotinic acid, ascorbic acid and α-tocopherol acetate were generously supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey, through the courtesy of Dr. J. C. Bauernfeind. Pteroylglutamic acid was supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. T. H. Jukes, and inositol by the A. E. Staley Manufacturing Company, Decatur, Illinois.

which is given in table 1. Sulfasuxidine [2] was included in the diet at the rate of 2 gm per liter of diet to inhibit intestinal synthesis of vitamin B_{12}. Individual feed and weight records were kept throughout the experiment.

After a 21-day depletion period, blood samples were taken by heart puncture and bone marrow samples by sternal aspiration.

One pig died during the depletion period. The remaining 12 pigs were divided into three groups of 4 animals each, according to weight, litter, and condition. During the 35-day

[2] Sulfasuxidine (succinylsulfathiazole) was generously supplied by Sharp and Dohme, Inc., Philadelphia, Pennsylvania, through the courtesy of Dr. S. F. Scheidy.

curative period the pigs in group 1, the control group, received no supplementation. Two of these pigs died of the deficiency, one during the third and one during the 4th week on experiment. Those in group 2 received intramuscularly 2 μg of crystalline vitamin B_{12} [3] daily; those in group 3 received intramuscularly 0.10 ml (2 units) of a commercial liver extract [4] daily.

This level of liver extract should have given a maximum response when injected, since optimum growth was previously obtained when 0.25 ml were administered orally. The extract was found to contain 19 μg of vitamin B_{12} per milliliter by the *L. leichmannii* assay procedure of Skeggs et al. ('48). At the completion of the curative period, blood and bone marrow samples were again taken.

When the pigs were sacrificed, the organs listed in table 3 were examined macroscopically. The spinal cord, brain, and a piece of sciatic nerve were also carefully removed from each pig and fixed in 10% formalin. Histological sections from these specimens were stained using the Weigert-Pal technique, the osmium tetroxide method, the Cajal silver method, Sudan III, and Spielmeyer's method for myelin sheaths in frozen sections (Carleton and Leach, '47). Small pieces of thyroid, spleen, kidney, liver, skeletal muscle, tongue, digestive tract, and bone marrow were fixed in Bouin's fluid. After being embedded in paraffin, these tissues were sectioned at 7 μ and stained with Harris's haematoxylin and eosin.

RESULTS

The average growth data of the three groups of pigs are shown in table 2. No differences were found between groups 2 and 3. However, the two pigs in group 1 (the control group) which lived grew at a significantly slower rate ($P = < 0.001$) than did the pigs in groups 2 and 3.

[3] Generously supplied by Merck, Inc., Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

[4] Reticulogen. Generously supplied by Eli Lilly and Co., Indianapolis, Indiana, through the courtesy of Dr. E. C. Kleiderer.

TABLE 2

Performance of pigs on crystalline vitamin B_{12} and on reticulogen

ITEMS COMPARED	GROUPS AND TREATMENT		
	I Unsupplemented basal diet	II 2 μg B_{12} daily after 21st day	III 0.10 ml reticulogen daily after 21st day
Number of pigs	2 [1]	4	4
Ave. initial wt. (kg)	1.42	1.30	1.32
Ave. final wt. (kg)	7.24	15.54	15.52
Ave. daily gain (kg)	0.104	0.254 [2]	0.254 [2]
Ave. final wt. as multiples of initial wt.	5.04	12.47	12.15
Kg dry matter consumed per kilogram gain	1.55	0.98 [3]	0.98 [3]

[1] Three pigs died on the deficient diet. Data not included.
[2] Significant over group I ($P = < 0.005$).
[3] Significant over group I ($P = < 0.001$).

TABLE 3

Mean weights of organs from pigs on vitamin B_{12}, reticulogen tests
(Expressed as percentage of body weight)

ORGANS EXAMINED	GROUPS AND TREATMENT		
	I Unsupplemented basal diet (1 pig)	II 2 μg B_{12} daily after 21st day (3 pigs)	III 0.1 ml ret. daily after 21st day (3 pigs)
Thymus	0.099	0.164	0.175
Heart	0.554	0.498	0.487
Thyroid	0.138	0.064	0.075
Lungs	0.811	0.828	0.789
Spleen	0.079	0.135	0.117
Kidneys	0.776	0.544	0.686
Adrenals	0.020	0.028	0.025
Liver	4.280	2.710	3.070
Tongue	0.891	0.384	0.424
Omentum	Fine, thin, little fat	Fine, thin, little fat	Fine, thin, some fat
Marrow sternum	Dry, non-fluid	Purple, cellular	Purple, cellular
Vertebrae	Dry, non-fluid	Purple, cellular	Purple, cellular
Femur	Dry, non-fluid fibrous matrix	Purple, cellular	Purple, cellular

The average growth curves of the three groups are given in figure 1. There was an immediate growth response in groups 2 and 3 after supplementation was begun.

Table 3 shows the mean organ weights, expressed as a percentage of body weight, of the three groups of pigs. Among the differences found was the increased size of the tongue

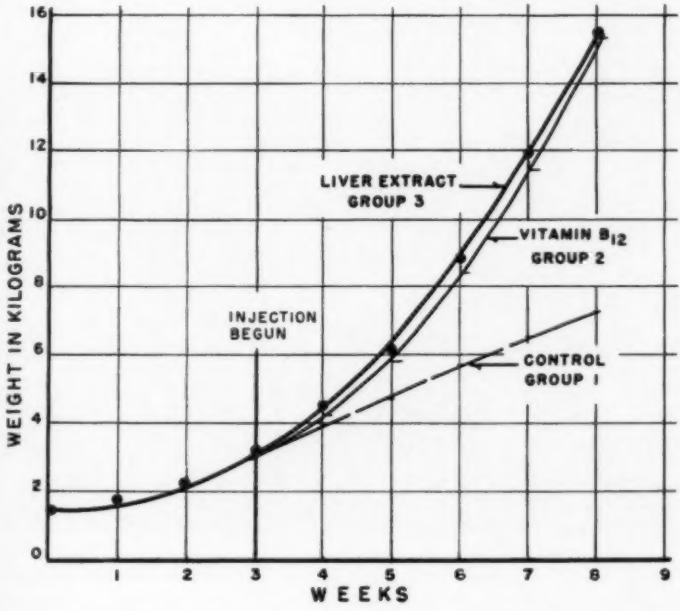

Fig. 1 Average growth curves of the baby pigs: effect of crystalline vitamin B_{12} and of antipernicious anemia liver extract (Reticulogen).

and liver in the pig of the control group examined. These differences, judging by the application of Chauvenet's criterion (Davenport and Ekas, '36) are sufficiently extreme to permit the conclusion that the control animals probably were not members of the same populations as the two treated groups. The tongue of the control pig was enlarged and reddened. On microscopic examination of the anterior part

of the tongue of this unsupplemented pig it was found that the papillary bodies of the squamous cell layers reached close to the surface of the tongue and were highly vascularized and diffusely infiltrated with leukocytes. This vascularized granulomatous tissue extended into the submucosa and formed a distinct layer between muscular coat and covering mucosa (figs. 2 and 3). Similar but far less extensive lesions were seen in the tongues of other animals.

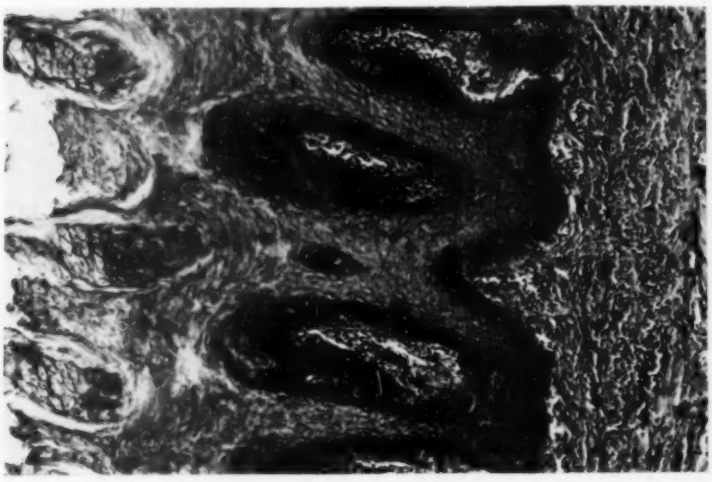

Fig. 2 Section of the anterior part of the tongue from an unsupplemented pig. × 90.

Enlargement of the liver without enlarged spleen has been reported by Zuelzer and Ogden ('46) in megaloblastic anemia of the human infant, which responded to folic acid or liver extract.

The mean blood values are given in table 4. There are no apparent differences between groups with regard to the red blood cell series. The distribution of neutrophils and lymphocytes was abnormal at the end of the depletion period. In groups 2 and 3 the distribution became normal after therapy,

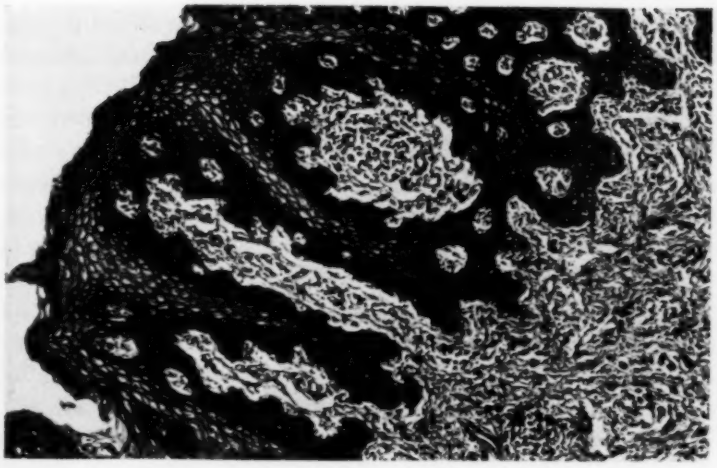

Fig. 3 Section of the anterior part of the tongue from a pig receiving B_{12}. Note the normal appearance of this tongue. × 90.

TABLE 4

Mean blood values from pigs on vitamin B_{12}, reticulogen tests
(Sample A taken on 21st day, sample B on 56th day)

	GROUPS AND TREATMENT					
	I Unsupplemented basal diet		II 2 μg B_{12} daily after 21st day		III 0.1 ml ret. daily after 21st day	
No. of pigs	4	2	4	4	4	4
Sample number	A	B	A	B	A	B
Hematocrit	..	43.0	..	44.1	..	43.4
Hemoglobin (gm/100 ml)	8.7	10.6	9.7	11.2	9.0	10.7
Red blood cells (millions/mm³)	5.0	7.49	5.2	6.98	4.3	6.50
Mean corpuscular volume (μ³)	..	57.2	..	63.1	..	66.1
Mean corpuscular hemoglobin (μμg)	..	47	..	49	..	46
Mean corpuscular hemoglobin concentration (%)	..	24	..	25.5	..	24.8
Leukocytes (thousands/mm³)		12.8	13.0	14.0	13.9	15.5
Neutrophils (%)	52	84	62	20[1]	61	14[1]
Lymphocytes (%)	42	15	34	79	37	85
Monocytes (%)	2	0	2	0	1	0
Eosinophils (%)	1	1	0	1	1	1

[1] Significantly different to rest of neutrophil percentages ($P = < 0.01$).

whereas in the control group the percentage of neutrophils increased still further with a concomitant decrease in lymphocytes.

The bone marrow data are given in table 5. The experimental pigs when compared with the normal farm pigs showed a significant decrease in the myeloid series and a concomitant increase in the erythroid series, while on the unsupplemented diet. Following vitamin B_{12} or liver extract injection, there was a decrease in the erythroid series in

TABLE 5

Mean bone marrow values before and after crystalline vitamin B_{12} or reticulogen injection

	GROUPS AND TREATMENT						
	Pigs on natural diets [1]	I Unsupplemented basal diet		II 2 μg B_{12} daily after 21st day		III 0.1 ml ret. daily after 21st day	
		A	B	A	B	A	B
No. of pigs	5	2	1	3	1	3	1
Total cells/mm³	180						
Per cent myeloid cells	76 [2]	57	20	40	71 [2]		69 [2]
Per cent erythroid cells	23	40	78	47	13 [3]		15 [3]
Per cent lymphoid cells	1	3	2	13	16		16
Differential of myeloid series:							
Mature neutrophils	36	64	40	54	73		69
Metamyelocytes	22	26	26	34	16		13
Myelocytes	29	7	30	10	8		10
Myeloblasts	1	1	0	1	0		4
Eosinophils and basophils	12	2	4	1	3		4
Differential of erythroid series:							
Normoblasts, orthochromic	86	94	50	78	74	Sample not suitable	76 76
Normoblasts, basophilic	13	4	50	20	21		21
Normoblasts, macrocytic	1	2		2	3		3
Erythroblasts			present				

[1] Samples taken at 56 days from pigs fed on natural farm diets plus pasture on the University Swine Farm.

[2] Significantly higher than the controls.

[3] Significantly lower than the control pig or the pigs before therapy ($P = < 0.01$).

the two pigs examined. The bone marrows of all pigs were cellular. With no differences in the peripheral blood with respect to hemoglobin concentration and red cell count it appears that the pigs were just able to produce the necessary number of red cells without anemia, the efforts of the erythroid series being reflected in the high percentage of nucleated red cells in the marrow. Primitive immature forms, as in pernicious anemia, were not observed under the experimental conditions and period of observation employed. The differential counts of the myeloid series showed a higher eosinophilia in the farm pigs but no other significant differences.

In the differential counts of the erythroid series the one pig examined after 8 weeks on the basal ration had a greatly increased number of basophilic normoblasts compared to either the sow-fed pigs or to the experimental pigs receiving vitamin B_{12} supplements. In all marrows a very small number of primitive large erythroblasts were seen. These cells had a large blue cytoplasm and an oval-shaped nucleus with a distinct nucleolus, and the nuclear chromatin showed a coarse network. These cells resembled closely the "megaloblast-like cells" described by Cartwright and Wintrobe ('49). They were found in farm control animals as well as experimental animals and never increased above 1% of the nucleated red cell series in this experiment.

Three of the pigs on the vitamin B_{12}-deficient ration died during the experiment, one during the depletion period and two in the control group. The latter two showed evidence of posterior incoordination. However, no demyelination was found in sciatic nerve or spinal cord at 5 different levels. On microscopic examination only a slight thickening of the tip of the villi in the ileum and colon was detected. This was not regarded as a significant lesion. The vitamin B_{12}-deficient pigs were listless and sluggish in their movements, especially during the latter half of the 8-week experiment.

DISCUSSION

The data presented in this paper demonstrate that the missing factor in the synthetic milk diet which we have developed is vitamin B_{12}, and that this is the factor supplied by injectible liver extract. Crystalline B_{12} and injectible liver extract [5] gave equal responses when injected in the baby pig at a level which was presumably above the optimum for growth. Ott et al. ('48) and Lillie et al. ('48) have demonstrated that crystalline vitamin B_{12} replaces the "animal protein factor" and the "cow manure factor" as well as liver extracts for chick growth. Hoffmann et al. ('48) have reported that crystalline vitamin B_{12} and liver extract produce the same maximum growth response with *L. leichmannii*.

Smith ('48) concentrated from liver two related red pigments, both hematopoietically active in pernicious anemia but differing from each other in that one was acidic and the other neutral. Similarly, it is possible that purified liver extract may contain another factor similar to vitamin B_{12} and having equal potency in our basal diet.

The most apparent blood abnormality in the pigs raised on the vitamin B_{12}-deficient diet was the high neutrophil count and concomitantly low lymphocyte count in the peripheral blood. This blood picture has been observed on this basal ration in three different experiments with pigs, and is in contrast to the marked granulocytopenia of pernicious anemia in man and of PGA deficiency in man and rats. The blood picture also differed from that in pernicious anemia in that there was only a slight normocytic anemia and no leukopenia.

Megaloblasts have not been found in the bone marrow of the pigs on the B_{12}-deficient diet, although they are typical of pernicious anemia. Heinle, Welch and Pritchard ('48) have reported macrocytic anemia with megaloblastic hyperplasia of the bone marrow in swine on a diet essentially free

[5] See footnote 4, page 405.

of the extrinsic factor and PGA and containing a crude PGA antagonist and succinylsulfathiazole. They, however, used older animals which remained on the basal ration for a longer period of time; and in addition they were dealing with a dual deficiency, whereas our work had to do with a vitamin B_{12} deficiency only.

The tongue lesions observed need further study to determine their specificity.

The absence of nerve lesions in the spinal cord and sciatic nerves of the pigs on the B_{12}-deficient diet was surprising in view of the gross symptoms of posterior incoordination, unsteadiness of gait, and so forth. However, the neuromuscular junctions were not examined.

SUMMARY

Crystalline vitamin B_{12} has been shown to be required by the baby pig. This vitamin and a pernicious anemia liver extract, when injected to supply approximately equal amounts of vitamin B_{12} potency, produced equal responses in growth rate. The equality in growth response indicates that vitamin B_{12} is the growth factor being supplied by the antipernicious anemia liver extract. The relation between the deficiency produced in baby pigs and pernicious anemia in man is discussed.

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FACTORS AFFECTING THE STABILITY OF THE VITAMIN A FROM COD LIVER OIL IN CEREAL FEEDS[1]

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Numerous investigators have studied the losses of vitamin A in foods and feeds containing added cod liver oil. Marcus ('31) showed that vitamin A is unstable when added to finely divided solids. Approximately 85% of the vitamin, as measured by the Carr-Price reaction, was lost in 10 days when the unsaponifiable fraction was added to a vitamin A-free rat ration (U.S.P. basal diet) or to granulated lactose. No difference in vitamin A destruction was noted between storage under air or carbon dioxide. Fraps and Kemmerer ('37) reported that 79 to 100% of the vitamin A (cod liver oils) added to white corn meal was destroyed after 4 weeks' storage in the presence of air at either 7 or 28°C. They determined the vitamin A content of the stored samples by the spectrographic method.

Holder and Ford ('39) and Bethke et al. ('39) measured vitamin A losses, in a mixed ration containing added cod liver oil, by the chick assay method.[3] The ration was stored in burlap sacks at room temperature (21 to 27°C.). Holder and Ford found no loss of vitamin A during storage periods of 8

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[3] Growth rate, deficiency symptoms and mortality records were used as criteria for comparison between groups of chicks.

weeks or less but were able to demonstrate a slight loss in a sample which was stored for 10 weeks. Bethke et al. observed that about 50% of the vitamin A activity of cod liver oil was still present in the ration after 6 months' storage. They further showed that the rate of destruction of vitamin A was approximately doubled by substituting meat scraps and dried skim milk for the casein component of the ration.

Thus, the data in the literature show ample proof of the instability of the vitamin A of cod liver oil in feeds and rations. However, the factors which retard or prevent the destruction noted have not been adequately investigated, and no explanations for the observed discrepancies are evident.

It was shown earlier in this laboratory that the carotene of dehydrated alfalfa can be effectively stabilized by sealing (in air) if the moisture content of the enclosed alfalfa is adjusted to 8 to 10% (Halverson and Hart, '48). Successful experiments with carotene added to mixed rations as alfalfa meal (15%) were also carried out. The observed stabilization was shown to be dependent upon the ability of the sealed alfalfa and cereal products to use up quickly the enclosed oxygen by oxidative reactions (Bailey and Gurjar, '20; Bailey, '40; Snow and Wright, '45; Halverson and Hart, '47).

Previous work in this laboratory has shown that the addition of free trace minerals (Fe, Cu, Co and Mn) to mixed rations containing alfalfa meal has no effect upon the observed carotene preservation that is obtained with feeds sealed with moisture contents of 8 to 10% (Halverson and Hart, '48). Also, the expected rancidity and bleaching changes which normally develop in such rations are completely prevented by sealing the stored feeds.

EXPERIMENTAL

Experiments were introduced to determine whether the vitamin A of cod liver oil is stabilized as effectively as is the carotene of alfalfa in feeds stored in gas-tight containers. Measured levels of cod liver oil were added to a mixed ration and to ground white corn samples which were stored under

sealed and unsealed conditions and at different moisture levels (3.2 to 15%). A commercial cod liver oil which contained approximately 290 μg of vitamin A alcohol per gram was employed throughout the studies. The percentage composition of the mixed ration follows: white corn, 30; wheat middlings, 20; wheat bran, 10; soybean meal, 20; oats, 16; $CaCO_3$, 2; $Ca_3(PO_4)_2$, 1; and iodized salt, 1. Cod liver oil was added to this ration at levels of 0.5, 1 and 2%. All of the mixed ration samples were stored at 33 to 36°C. The effect of different storage temperatures upon vitamin A stability under sealed conditions and at different moisture levels was studied in an experiment with ground white corn which contained 1% of added cod liver oil. The white corn samples were prepared in duplicate to enable storage at two temperatures (22 to 25°C. and 33 to 36°C.). After 2.5 months or more of storage, the mixed ration and white corn samples were opened and analyzed for vitamin A and the percentage loss in the different samples was calculated.

In order to facilitate convenient adjustment of the moisture content of feeds, they were initially dried for approximately two hours at 95°C. prior to addition of the cod liver oil. Weighed portions of cod liver oil were then mixed into the dried feeds after they had been allowed to cool to room temperature. The feeds were then stored in glass chambers until moisture analyses could be made. The moisture content of portions of the feed samples containing added cod liver oil was then adjusted to definite levels and the samples were placed in pint-size ice cream cartons (3 × 4 inches). The feeds were firmly packed into the containers by hand. The filled cartons, which contained 300 to 350 gm of feed apiece, were then thoroughly coated with a commercial wax [4] by intermittent dipping in the melted wax.

The respiratory activity of the mixed ration containing no added trace minerals was determined at different moisture

[4] The Flexowax employed in these studies was obtained from the Glyco Products Company, Inc., Brooklyn, New York. This wax melts at 52°C., is fairly flexible and does not readily crack.

levels (7.5 to 20%). The mixed ration employed was of the same composition as that used in the vitamin A stabilization studies except that no cod liver oil was added. One-hundred-gram samples of mixed ration (dry basis) were adjusted to the proper moisture content and then enclosed in gas-tight glass receptacles. The samples were stored in the dark for 5 days at 22 to 25°C. The changes in composition of the enclosed air were followed by determining the carbon dioxide and oxygen contents of the enclosed atmosphere at the end of the storage period. These contents were expressed as volume per cent of the enclosed gas. A detailed description of the apparatus and techniques employed in determining the effect of moisture level upon the respiratory activity of feed samples is given by Halverson ('49).

Since small amounts of both the trace minerals and vitamin A (fish liver oils) are commonly added to commercial poultry and certain livestock feeds, an investigation of the effect of these minerals upon the destruction of the added vitamin A in different feeds seemed desirable. A mixed ration containing added trace minerals and cod liver oil was stored under sealed and unsealed conditions and at different moisture levels for 10.5 months at 33 to 36°C. This ration was of the same composition as the mixed ration already described except for the presence of the added trace minerals. The following minerals were added in grams per 100 gm of ration: $FeCl_2 \cdot 4H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.02; and $MnSO_4 \cdot H_2O$, 0.25. The minerals were finely ground in a mortar before being mixed into the ration.

An additional experiment was carried out to obtain further information about the effect of the trace minerals upon the rate of vitamin A destruction. In this instance ground white corn samples containing added cod liver oil were stored in contact with air (in paper sacks) and at 36 to 39°C. Vitamin A analyses were made at 0-, 3-, 6-, 12- and 24-day intervals and the percentage loss in the different samples was calculated.

In the same experiment an attempt was also made to find a convenient way to prevent the trace minerals from coming into

intimate contact with the feed as well as the added vitamin A. The idea of dissolving the minerals in gelatin seemed worthwhile; therefore, a gelatin water solution was prepared by mixing two parts of hot water containing the dissolved trace minerals with one part of gelatin flakes. After the heated solution was thoroughly mixed, it was allowed to cool and then dried in a vacuum desiccator at room temperature for 24 hours. After an additional drying period of 36 hours at 50°C. in a vacuum oven, the gelatin-mineral mixture was dry.

The same kind and amount of minerals were employed with the white corn as with the mixed ration experiment. The following amounts of the trace minerals in grams were dissolved in 2 gm of gelatin: $FeCl_2 \cdot 4H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.02; and $MnSO_4 \cdot H_2O$, 0.25. Thus, the addition of the above amount of minerals to 100 gm of ground white corn involved the addition of 2 gm of carrier gelatin. The dried gelatin-mineral mix was ground in a Wiley mill, using a 2 mm sieve. In order to obtain uniform particles of different size, sieves were used to obtain two major fractions — one fraction passing through an 0.85 mm square mesh sieve and the other through a 1.5 mm round mesh but not through an 0.85 mm square mesh sieve. Equivalent amounts of gelatin were added to all of the corn-cod liver oil samples, whether the additions were made as the gelatin-mineral mix or merely as gelatin flakes and whether they were added to samples containing no added trace minerals or the trace minerals added in free form (unprotected).

Vitamin A analysis

The method of vitamin A analysis employed was essentially that described by The Association of Vitamin Chemists, Inc. ('47). The vitamin A was extracted from the samples with ether. After saponification the washed ether extract was freed of excess water by shaking with portions of saturated aqueous NaCl solution, rather than by treatment with anhydrous Na_2SO_4. The blue color of the Carr-Price reaction was meas-

ured with the Evelyn colorimeter and calculations were made following calibration of the instrument with the U.S.P. Vitamin A Reference Standard, 1948.

During the latter phases of the study, a chromatographic purification was included in the analytical procedure to remove further the colored pigments present in the saponified extract. In the mixed feed analysis, a high blank reading was always obtained with samples containing no added vitamin A. The error introduced by blank corrections was not too great, but the possibility that foreign substances or pigments might be inhibiting color development in the antimony trichloride reaction was always apparent. Any procedure capable of reducing the blank reading and, thereby, removing impurities would thus give additional information to substantiate or disprove the results.

The chromatographic column employed consisted of a mixture of 5 parts of Hyflo Super-Cel [5] and 3 parts of lime.[6] The column, which was 19.4 mm in diameter by 10 to 11 cm long, was firmly packed under partial vacumm and covered with a 1- to 2-cm layer of anhydrous Na_2SO_4. This column gave recoveries for pure vitamin A (saponified) of 95 to 97% and for vitamin A added to feed extracts of 90 to 95% under the conditions employed in the experiments. Thus, it was concluded that any inhibitory substances that might originally have been present were removed by the column, since good recoveries were obtained even in the presence of extracts from the mixed ration. Since the blank readings were also reduced to negligible values, it was possible to make the calculations with reasonable confidence that the blank corrections were not introducing a significant degree of error.

The procedure employed for the chromatographic determinations was as follows. The purified unsaponifiable extract was dissolved in petroleum ether (distilled Skelly A) by evaporating off the ether solvent and replacing it with Skelly A. After the adsorption column was completely wet with Skelly

[5] Johns-Manville, Chicago, Ill.

[6] Grand Prize Hydrated Finishing Lime, United States Gypsum Co.

A, the vitamin A extract was poured onto the column and suction was applied. The adsorbed vitamin A was eluted immediately with 200 ml of a solution of 3% acetone in Skelly A. The entire eluant, together with the extract solvent, was collected in a single fraction. After the Skelly A solvent was evaporated off, the chromatographed residue was dissolved in a measured volume of dry distilled chloroform, and then colorimetric determinations (Carr-Price) were made by the standard procedure.

The mixed ration analyses were performed by the standard Carr-Price method as described by The Association of Vitamin Chemists, Inc. ('47), as well as by the extended chromatographic procedure. The two methods gave values which agreed with surprising uniformity, the reported values being obtained by the standard method. The samples reported in tables 2 and 4 were analyzed only by the extended chromatographic procedure. All samples were analyzed in duplicate and the reported values represent the average of the two analyses. Good agreement was obtained between the duplicate analyses, for the average variation between different analyses of the same sample was generally not greater than 10%.

RESULTS

The data show that sealing to prevent the access of oxygen as well as to permit control of the moisture level had little effect upon the retention of the vitamin A of cod liver oil added to a mixed ration or ground white corn samples. The losses for the mixed ration are shown in table 1 and those for the white corn in table 2. Samples sealed with high moisture levels (10 to 15%) showed no greater preservation during storage than those at lower moisture levels (3.3 to 5%) or even unsealed samples. Thus, it is obvious that the absence of oxygen as induced by respiratory action (table 3) at the higher moisture levels was not the primary factor in vitamin A preservation in the samples studied.

Significant losses of vitamin A occurred during storage under sealed and unsealed conditions and at different moisture

levels. While slightly greater losses were apparent in the unsealed than in the sealed mixed ration samples when the storage period was 9 to 10.5 months at 33 to 36°C., no definite

TABLE 1

Vitamin A losses in a mixed ration sealed at different moisture levels and with and without added trace minerals. (Storage temperature 33–36°C.)

DESCRIPTION OF SAMPLE	WATER CONTENT	NO ADDED TRACE MINERALS			ADDED TRACE MINERALS
		2.5 mos.[1]	3 mos.[2]	9 mos.[3]	10.5 mos.[4]
	%	% loss	% loss	% loss	% loss
Unsealed	5.1	44	38	92	100
Sealed	3.3	34	36	54	90
Sealed	5.0	34	36	66	92
Sealed	7.5	44	44	73	91
Sealed	10.0	36	37	73	90
Sealed	12.5	41	33	..	91
Sealed	15.0	31	47	..	89

[1] One per cent of cod liver oil in ration (290 μg of vitamin A alcohol per gram of cod liver oil).

[2] Five-tenths per cent of cod liver oil in ration.

[3] Two per cent of cod liver oil in ration.

[4] Five-tenths per cent of cod liver oil in ration containing the following percentages of trace minerals: $FeCl_2 \cdot 4H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.02; and $MnSO_4 \cdot H_2O$, 0.25.

TABLE 2

Effect of different storage temperatures upon vitamin A losses in stored white corn sealed at different moisture levels[1] (8-month storage period)

DESCRIPTION OF SAMPLE	WATER CONTENT	PER CENT OF LOSS AT	
		22–25°C.	33–36°C.
	%		
Unsealed	7.1, 5.0	26	39
Sealed	3.2	20	22
Sealed	5.0	20	26
Sealed	7.5	17	28
Sealed	10.0	18	26
Sealed	12.5	25	39
Sealed	15.0	30	46

[1] One per cent of cod liver oil added to the ground white corn samples (290 μg of vitamin A alcohol per gram of cod liver oil).

differences in preservation could be credited to sealing with either the white corn samples after 8 months' storage at two different temperatures or the mixed ration samples after 2.5 to 3 months' storage at 33 to 36°C. Vitamin A losses were greater in the sealed corn samples which contained 12.5 and 15% of moisture than in those sealed with lower water content.

The extent of the vitamin A losses appeared to be dependent upon the sample composition as well as the length and temperature of storage, rather than upon sealing and control of the moisture level of the sealed samples. Losses with the mixed ration containing added cod liver oil were more rapid than

TABLE 3

Effects of moisture content upon carbon dioxide production and oxygen consumption by the mixed ration containing no added trace minerals.[1] *(Stored for 5 days at 22 to 25°C.)*

MOISTURE CONTENT OF SAMPLE	CO_2	O_2
%	%	%
7.5	0.2	19.5
10.0	1.0	18.9
12.5	2.5	14.7
15.0	4.3	5.4
20.0	11.8	0.2

[1] All samples initially sealed in air.

those observed with white corn; slightly greater losses resulted with the former in 2.5 to 3 months than with the latter in 8 months' storage. Larger losses of vitamin A were also observed in mixed ration samples stored for 9 months than were found after 2.5 to 3 months of storage under either sealed or unsealed conditions (table 1). The higher storage temperature employed in the white corn studies shown in table 2 similarly resulted in an accelerated rate of vitamin A destruction in both the sealed and unsealed samples.

Most of the vitamin A was destroyed in the mixed ration during 9 to 10.5 months' storage under sealed and unsealed conditions, and with and without the added trace minerals

(table 1). Because the loss was so extensive in the sample without added trace minerals (Fe, Cu, Co and Mn) when the storage period was 9 months, it is obvious that a shorter period of storage is essential for studying the assumed capacity of the trace minerals to accelerate vitamin A destruction in sealed samples.

The ability of the trace minerals to accelerate the destruction of vitamin A in unsealed samples was demonstrated by the results shown in table 4. Almost complete vitamin A de-

TABLE 4

Vitamin A losses in stored white corn (unsealed) containing the trace minerals added in free form and in a dried gelatin-mineral preparation.[1]
(Storage temperature 36–39°C.)

DESCRIPTION OF SAMPLE	% LOSS DURING STORAGE PERIODS OF			
	3 days	6 days	12 days	24 days
Sample (corn + vitamin A) + gelatin flakes	0	0	24	37
Sample + coarse gelatin-mineral mix	1.2	13	25	40
Sample + fine gelatin-mineral mix	1.6	14	39	56
Sample + gelatin flakes + free trace minerals	76	92	95	98

[1] One per cent of cod liver oil added to the ground white corn samples (290 μg of vitamin A alcohol per gram of cod liver oil).

struction occurred in 6 days in a white corn-cod liver oil sample which contained added trace minerals. The samples reported in table 4 were stored in the presence of air (in paper sacks) at 36 to 39°C. The stabilizing effect of adding the minerals (Fe, Cu, Co and Mn) in the form of a dried gelatin preparation was apparent. The vitamin A loss for the sample containing the coarse gelatin-mineral mix was not significantly greater than for the sample which contained no added trace minerals. While the observed loss in the sample containing the fine gelatin-mineral mix was more rapid than in the coarse gelatin-mineral sample, the preservation obtained with the

former was still very pronounced when compared with the sample which contained the trace minerals in free form (unprotected).

The white corn sample containing the trace minerals in free form developed a rancid odor as well as a yellow gold color within a few days of storage at 36 to 39°C. No changes in odor or color were evident in the corn plus added vitamin A samples which contained either no added trace minerals or else the minerals added as the coarse gelatin-mineral mix. Very slight rancidity and color change were evident in the corn sample containing the fine gelatin-mineral mix after 24 days' storage.

DISCUSSION

The studies reported indicate that vitamin A losses in a mixed ration and white corn samples containing added cod liver oil are not affected by the presence of gaseous oxygen. The fact that the type of feed employed (mixed ration versus white corn) affected the rate of vitamin A loss, while sealing did not, indicates that the presence or absence of undefined constituents in a feed has more effect upon vitamin A destruction than does the presence of oxygen. It is interesting that sealing in gastight containers prevented the development of rancidity (color and odor changes) and yet had no effect upon vitamin A losses.

It was notable that the rapid vitamin A destruction and rancidity development in the white corn sample containing added free trace minerals (Fe, Cu, Co and Mn) were largely prevented when the minerals were added in the form of a gelatin-mineral mix rather than in free form. Thus it is apparent that such minerals can be added to rations in an available form by which the usual destructive catalysis of fats, vitamin A and other similar compounds is almost completely prevented. The ratio of gelatin to minerals employed in the study was about 6.5 to 1.

The results with the mixed ration (unsealed and without added trace minerals) are in essential agreement with those obtained by Holder and Ford ('39) and Bethke et al. ('39), who measured storage losses of vitamin A in a mixed ration

by the chick assay method. These investigators observed slow losses of vitamin A during the extended storage (in open sacks) of mixed rations which contained no added trace minerals. They were unable to obtain the rapid losses noted by Fraps and Kemmerer ('37) in spectrographic studies with white corn containing added cod liver oil.

SUMMARY

1. The vitamin A in a mixed ration and ground white corn samples containing added cod liver oil was not stabilized by storage in sealed containers (gas-tight) at different moisture levels. The removal of enclosed oxygen by respiration of the feed samples at higher moisture levels (10 to 15%) showed no demonstrable effect upon vitamin A preservation. Comparable vitamin A losses occurred in sealed and unsealed samples under similar storage conditions. Vitamin A losses in both sealed and unsealed samples increased with temperature and length of storage.

2. Significant amounts of the added vitamin A in the mixed ration and white corn samples (without added trace minerals) were retained after several months' storage. Approximately 60% of the initial vitamin A content of the mixed ration was still present in samples stored in sealed and unsealed containers for 2.5 to 3 months at 33 to 36°C. The vitamin A of cod liver oil was more stable in ground white corn than in the mixed ration, under sealed and unsealed conditions and at similar storage temperatures.

3. The extremely rapid destruction of vitamin A induced by adding trace minerals (Fe, Cu, Co and Mn) to an unsealed white corn sample containing added cod liver oil was largely prevented by adding the minerals in a dried gelatin-mineral mixture rather than in free form. The experiments did not determine whether the free trace minerals are capable of accelerating vitamin A destruction in feeds stored in an atmosphere free of oxygen.

4. The effective prevention of rancidity and vitamin A destruction in practical poultry rations and certain livestock

feeds which commonly contain small amounts of added (free) trace minerals is an important problem. The present study indicates that a practical solution of the problem depends upon the addition of the trace minerals in a form which limits their ability to come in contact and react with other constituents of the ration.

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FATTY ACID CHANGES IN EGG YOLK OF HENS ON A FAT-FREE AND A COTTONSEED OIL RATION [1]

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Available evidence indicates that the production and hatchability of eggs and the mortality of embryos are unaffected by the level of fat in the ration if the level does not go below 1% (Taylor et al., '44; Heywang, '43) but may be affected if the level is below 1% (Davis and Upp, '41). Thus, as has been shown by Russell and co-workers ('41), a producing hen on a low fat intake deposits in the egg considerably more fat and (McCollum and co-workers, '12) more phospholipid than it consumes. Reliable analyses by Riemenschneider et al. ('38) have shown that both the phospholipid and triglyceride fatty acids contain over 8% linoleic acid, and that the triglyceride acids contain over 2% and the phospholipid acid over 13% "clupanodonic" acid.

It has been repeatedly demonstrated, however, that on a low fat diet the iodine number of yolk acids decreases (Russell et al., '41; McCollum et al., '12; Riemenschneider et al., '38). Since this decrease in iodine number is probably due to the loss of polyunsaturated acids, the question arises as to whether the quantity of these acids will reach a minimum or whether they will completely disappear from the yolks of

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eggs laid by hens on a fat-free ration. If the polyunsaturated acids continue in the yolk it would thereby be proved that the hen can synthesize them from non-fat precursors. Furthermore, a study of the production, fertility and hatchability of eggs containing no polyunsaturated acids would give new information on the requirements of poultry for the so-called essential fatty acids.

The present investigation was conducted in order to determine the effect of the rigid removal of fat from the rations of laying hens on the production, fertility and hatchability of eggs, and the mortality of embryos, and on the fatty acid composition of the egg yolk.

The hens were 7-month-old white leghorns just coming into production; they were placed in individual cages and supplied with running water. Four were placed on a synthetic fat-free ration and three on a 4% cottonseed oil[2] ration. Feed was given fresh each day. The hens were artificially inseminated weekly and alternate eggs incubated and analyzed. The yolks from each hen were pooled for two-week periods and moisture, total lipid, phospholipid and cholesterol determined. The yolks from each group not used for the above determinations were pooled, and the fat extracted and divided into acetone soluble and insoluble fractions. The fatty acids of each fraction were converted to methyl esters after removal of unsaponifiable matter. The methyl esters were analyzed spectrophotometrically for the polyunsaturated fatty acids and their total iodine value determined.

PROCEDURE

Rations

The fat-free ration consisted of 63.2% sucrose, 4% liver L,[3] 25% soybean protein, 5.0% salt mixture, 0.6% DL-methionine, 1.8% glycine, 0.25% choline, 0.125% *i*-inositol, and of alpha tocopherol acetate 30 mg per kilogram, crystalline vita-

[2] Wesson.
[3] Wilson's.

min D_3 0.025 mg per kilogram, and carotene 100 mg per kilogram. The water soluble vitamins were added in amounts recommended by the National Research Council. For the fatty ration, 4% cottonseed oil replaced 8% sucrose.

Analyses

1. Total lipid, cholesterol and phospholipid. Approximately 1 gm of yolk was extracted by adding about 0.5 ml of 3:1 alcohol-ether in a 50-ml beaker and mixing with a glass rod. Increasing amounts of the solvent mixture were added slowly to about 20 ml. This resulted in a very fine precipitate. The mixture was transferred quantitatively to a 100-ml volumetric flask with about 50 ml of the solvent. The solution was brought to a boil, cooled, made up to volume and allowed to stand overnight.

Total lipid was determined on a 50- or 75-ml aliquot by evaporating to dryness. It was unnecessary to re-extract, since all the alcohol-ether residue is soluble in petroleum ether. Total cholesterol was determined on a 1-ml aliquot by the method of Schoenheimer and Sperry ('34). Phospholipid was determined on a 1-ml aliquot by the method of Reiser ('47), except that "superoxol" was used instead of nitric acid and a sandbath instead of a microburner.

2. Separation of phospholipid and glyceride fatty acid esters. a. Extraction: At each two-week period the yolks from the hens on each ration were pooled. They were extracted by slowly adding 10 volumes of 3:1 alcohol-ether mixture while vigorously stirring. The mixture was filtered by suction or centrifuged. The residue was then washed three times with 6 volumes of ethyl ether. The washings were added to the alcohol-ether extract and evaporated under reduced pressure to remove ether and alcohol. The water-fat mixture was extracted with petroleum ether in a separatory funnel, and the ether extract dried over anhydrous sodium sulfate and finally evaporated under reduced pressure.

b. Separation of phospholipids: The dry residue (which was completely ether-soluble) was dissolved in about three times its volume of ether and transferred in a slow stream while being vigorously mixed, into 1 l of acetone for each 40 gm of mixed fat. Twenty-milliliter portions of a saturated solution of $MgCl_2$ in 95% alcohol were added during the transfer and while the mixture was constantly stirred. The phospholipid was allowed to precipitate over night. The clear acetone solution was decanted into a round-bottom flask with acetone washings of the insoluble material.

c. Preparation of methyl esters: *Phospholipid.* This procedure was patterned after that of Shinowara and Brown ('38). To each 10 gm of phospholipid were added 100 ml of 10% methanolic HCl and the mixture reflexed for 24 hours. About half of the alcohol was then evaporated under reduced pressure. The mixture was then transferred to a separatory funnel with petroleum ether and washed with water and sodium carbonate solution. The washed ether solution was dried over anhydrous sodium carbonate and evaporated to dryness under reduced pressure.

Glyceride. Because of the large amount of cholesterol, the unsaponifiable fraction had to be removed. Thus interesterification with methanol was impossible unless digitonin was used. This was tried but discontinued because of the high cost and the added manipulations required to recover the digitonin. Furthermore, the removal of cholesterol was not complete, possibly because of incomplete interesterification of cholesterol ester.

The procedure finally adopted was as follows: To each 10 gm of acetone soluble lipid warmed to 70°C. were added 6 ml of 50% KOH at the same temperature. After adding 150 ml of hot water, all the soaps were dissolved, by heating if necessary. The cooled solution was extracted 6 times with 20-ml portions of ethylene dichloride. The extracted soap solution was made acid with 10 ml of concentrated hydrochloric acid and boiled until a clear acid layer was obtained. The acids were dissolved in ether, washed with water, and

dried over anhydrous sodium sulfate. The final washing may be made with saturated NaCl to partial dryness. The ether was evaporated, three volumes of dry methanol containing 3% sulfuric acid added, and the solution reflexed for three hours. The bulk of the alcohol was boiled off. The esters were dissolved in ether, washed with water and sodium bicarbonate solution, dried and freed of ether. The esters contained traces of cholesterol but the amount was not considered large enough to interfere with subsequent analyses.

3. Determination of polyunsaturated fatty acids. Polyunsaturated fatty acids were determined according to the method of Brice, Swain, Schaeffer and Ault ('45). However,

TABLE 1

Fertility, hatchability, and mortality of embryos of eggs laid by hens on fat-free and cottonseed oil rations

	INCUBATED	HATCHED	INFERTILE	DEAD
Control period				
Fat-free group	10	7	3	0
CSO group	12	8	3	1
Test period				
Fat-free group	65	56	6	3
CSO group	41	24	10	7

because of the presence of 5 and 6 double bond acids and lack of standards it has not been possible to determine the amounts. Therefore absorption curves and standard absorption values at the wave lengths of maximum absorption were used comparatively.

OBSERVATIONS

With respect to production, fertility, and hatchability, there were no significant differences between the egg production of the hens on the fat-free or on the cottonseed oil rations. Unexpectedly, however, the eggs laid by the hens on the cottonseed oil ration had lower hatchability and fertility and the embryos a higher mortality than those of the hens on the fat-free diet (table 1).

TABLE 2

The lipid content of egg yolks produced by hens on fat-free and 4% cottonseed oil rations
(Values shown are in per cent)

HEN NO.	*Fat-free ration* 3117				3118				3119				3120			
WEEKS ON TEST	M[1]	TL[1]	P[1]	C[1]	M	TL	P	C	M	TL	P	C	M	TL	P	C
Control	48	71			47	76			46	78			49	71		
7 and 8	50	69	20.3		50	67	21.3		61	67	21.7		49	67	20.6	
9 and 10	50	66	22.5	2.88	49	63	22.3	3.12	50	69	22.9	2.92	49	67	22.8	3.26
11 and 12	50	69	20.8	2.91	50	67	21.0	3.38	50	72	20.8	3.16				
13 and 14	50	68	22.4	2.86	49	68	22.8	3.08	49	65	23.6	2.58				

HEN NO.	*Four per cent cottonseed oil ration* 3121				3123				3124			
WEEKS ON TEST	M	TL	P	C	M	TL	P	C	M	TL	P	C
Control	50	66			50	67			49	67		
7 and 8	50	69	20.7		49	67	21.9		50	69	21.0	
9 and 10	49	66	23.1	2.90	49	67	23.9	3.26	49	66	23.2	3.23
11 and 12	47	66	21.5	2.99					49	66	21.5	2.96
13 and 14	49	66	22.2	2.89	48	65	29.6	3.10	48	64	23.6	2.98

[1] M — moisture; TL — total lipid; P — phospholipid; C — cholesterol. All lipid values are on the dry basis.

The moisture, total fat, phospholipid and cholesterol content of the eggs of both groups remained unchanged during the entire course of the experiment (table 2).

The results of the spectrophotometric examinations of the polyunsaturated fatty acids are summarized in table 3. The figures given are absorption coefficients at 1 cm of a 1% solution of the methyl esters of the glyceride and phospholipid fatty acids. Because of the lack of constants for the 5 and 6 double bond acids it was not possible to calculate the percentage of the various acids present. The absorption coefficients, however, show some interesting relations.

When the hen's rations contained 4% cottonseed oil the 6 double bond acid content steadily declined, disappearing from the glycerides after 8 weeks and reaching one-third their original value in the phospholipids after 14 weeks. The content of 3, 4, and 5 double bond acids of both the glycerides and phospholipids, however, reached a minimum level only slightly below that of the control period in two weeks. The two double bond acids (linoleic) remained unchanged throughout in both the glycerides and phospholipids. There was a possible increase in the two and three double bond acids of the phospholipids after the 4th week.

The egg fatty acids of the hens on the fat-free ration underwent some noticeable changes. The 6 double bond acids disappeared from the glycerides after 4 weeks and the 5 double bond acids after 8 weeks. The three and 4 double bond acids decreased in 14 weeks to 25%, and the two double bond acids to 16% of the level of the control period, at which time they had not yet reached a minimum.

Because of the disappearance of the 5 and 6 double bond acids it is possible to calculate the glyceride fatty acid content of the yolks laid during the 13th and 14th weeks. They contained 0.46% of 4 double bond, 0.30% linolenic, 1.79% linoleic, 61.4% oleic and 36.2% saturated acids.

These figures may be compared to the composition of the glyceride acids on a "normal" ration as given by Riemenschneider, Ellis and Titus ('38). These workers found 2.3%

TABLE 3

The $E^{1\%}_{1\,cm}$ of the methyl esters of the glyceride and phospholipid fatty acids of the egg yolks of hens on fat-free and 4% cottonseed oil rations

TIME ON TEST RATION, WEEKS	GLYCERIDE ACID ESTERS					PHOSPHOLIPID ACID ESTERS				
	374 Mμ	347 Mμ	314 Mμ	268 Mμ	234 Mμ	374 Mμ	347 Mμ	314 Mμ	268 Mμ	234 Mμ
4% CSO ration										
Control	0.650	1.70	4.25	14.1	121	4.00	13.3	38.2	96.2	199
1 and 2	0.518	1.37	3.27	10.6	121	4.56	11.0	26.3	51.8	151
3 and 4	0.360	0.86	2.50	9.2	118	2.00	6.20	23.0	54.2	150
5 and 6	0.284	0.80	2.80	10.0	125	2.82	8.36	28.0	68.5	182
7 and 8	0.000	0.78	2.18	8.32	138	1.83	6.23	24.6	67.5	157
9 and 10	0.000	0.86	2.90	10.4	145	1.96	7.75	34.0	82.5	194
11 and 12	0.000	0.66	2.14	8.9	132	1.49	6.36	29.2	72.5	191
13 and 14	0.000	0.87	2.74	9.7	117	1.12	5.44	26.2	66.5	164
Fat-free ration										
Control	0.650	1.70	4.25	14.1	121	4.00	13.3	38.2	96.2	199
1 and 2	0.448	1.08	2.46	8.8	72	3.50	9.25	24.0	51.5	111
3 and 4	0.309	0.69	1.58	6.7	37	3.05	8.02	23.4	57.8	96
5 and 6						0.0	1.64	8.6	28.8	74
7 and 8	0.000	0.50	1.10	5.1	47	1.56	4.95	19.2	55.0	97
9 and 10	0.000	0.00	1.72	5.9	27	1.71	5.42	20.3	55.5	90
11 and 12	0.000	0.00	1.01	4.7	28	1.27	4.75	19.1	63.5	109
13 and 14	0.000	0.00	1.05	4.1	20	0.84	3.11	13.0	52.0	81

"clupanadonic," 8.6% linoleic, 55.7% oleic plus hexadecenoic, and 32.7% palmitic plus stearic acids.

The phospholipid fatty acids of the eggs from hens on the fat-free ration showed similar but more irregular changes. The 6 double bond acids decreased in 14 weeks to about 25% of the control level, the 5 double bond acids to about 33%. The 4 double bond acids decreased about 50% but the two and three double bond acids decreased comparatively little, if at all.

A disconcerting factor was an apparent increase in the unsaturated acids during the 7th to 10th weeks, especially noticeable in the phospholipid acids of eggs from hens on the fat-free ration but also noticeable in the glyceride acids. These acids reached a minimum value after 6 weeks on the experimental ration but during the next two-week period increased again before continuing the decrease. The explanation for this is not obvious. All rations were made from the same constituents and it does not seem possible that any fatty material could have inadvertently been allowed to enter them.

DISCUSSION

The apparent deleterious effect of cottonseed oil on the fertility and hatchability of eggs is an interesting observation in view of other adverse effects of this oil on the well-being of poultry which have been observed in this laboratory. It has been found, for example, to aggravate the effects of low riboflavin rations (Reiser and Pearson, '49).

The most significant results of the present study are the changes observed in the polyunsaturated acids (table 3). The disappearance or pronounced drop in the concentration of all polyunsaturated acids in the egg yolk of hens on a fat-free ration is a clear indication that the hen cannot synthesize these acids from non-fat precursors. It is significant that there was no decrease in production, fertility or hatchability as these acids slowly disappeared from the

eggs. It must not be concluded, however, that the hen has no requirements for fatty acid. The present experiment was not carried to the point of the complete disappearance of linoleic acid from the egg, and the body fat of the hens was not examined. By the same token it cannot be concluded that polyunsaturated acids are not required for the hatchability of eggs. As yet unpublished work in this laboratory indicates clearly that fat is essential to the growth of chicks.

The changes resulting from the presence of 4% of cottonseed oil in the basal fat-free ration prove that the 6 double bond acid or acids are not synthesized from either linoleic or oleic acids, since they disappear from the eggs at the same time on the cottonseed oil ration as when there is no fat in the diet. It is also clear that the 3, 4, and 5 double bond acids are synthesized from either the oleic or linoleic acid of cottonseed oil, or both, since these polyunsaturated acids, reaching a minimum value in the egg after two weeks on the cottonseed oil diet, maintained that level for the entire 14 weeks of the experiment. There are no linolenic or higher polyunsaturated acids in cottonseed oil.

The slow decrease during a 14-week period of 2 and 3 double bond acids in the eggs of hens on rations devoid of these acids shows that they must have been supplied from the depot fat of the hen. This is contrary to the finding of Almquist et al. ('34) that depot fat is not utilized for the production of yolk fat.

That the polyunsaturated acids of a ration may increase their concentration in the egg yolk has been demonstrated by Cruickshank, Houston and Moore ('39). These workers reported a pronounced increase in the absorption coefficient of egg fat at 2,300 and 2,700 Å when the hens were fed a fat containing polyunsaturated acids.

SUMMARY AND CONCLUSIONS

Two groups of hens were placed on a rigid fat-free and a 4% cottonseed oil ration, respectively. Alternate eggs were incubated and analyzed. No difference in egg production was

noted but fertility and hatchability and low mortality of embryos were favored by the fat-free ration.

Analyses of the egg yolks showed no change in moisture, total fat, phospholipid or cholesterol content.

Six double bond acids disappeared from the glycerides of the eggs of hens on the fat-free ration after 4 weeks and 5 double bond acids after 8 weeks. Two, three, and 4 double bond acids decreased more slowly reaching 16 to 25% of their control level in 14 weeks. The phospholipid acids showed similar changes.

On the same basal fat-free ration plus 4% cottonseed oil the 6 double bond acid disappeared at the same time as on the fat-free ration, but the 3, 4, and 5 double bond acids reached a minimum after two weeks. The two double bond acid remained unchanged.

It is concluded that the hen cannot synthesize polyunsaturated fatty acids from non-fat precursors, but that it can synthesize 3, 4, and 5, but not 6, double bond acids from linoleic (two double bond) acid.

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SOME INTERRELATIONSHIPS OF COPPER, MOLYBDENUM, ZINC AND LEAD IN THE NUTRITION OF THE RAT

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THREE FIGURES

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A knowledge of the interrelationships among nutrients in a diet is essential for an understanding of their quantitative requirements for the animal. Utilization of one nutrient may be profoundly affected by the presence or absence of another. Hart, Steenbock, Waddell and Elvehjem ('28) first demonstrated the animal's need of copper for the utilization of iron in hemoglobin formation. Moxan and Rhian ('43) found that arsenic reduced the toxic effect of selenium. Smith and Larson ('46) produced a zinc anemia in rats which was corrected by copper feeding. Ferguson, Lewis and Watson ('38) reported a scouring disease in England among cattle in areas in which the forage was found to have a high molybdenum content. Later analyses of this forage ('43) indicated normal copper levels. These workers reproduced the same symptoms in cattle experimentally by molybdate feeding and demonstrated that the disease could be cured by copper therapy. Britten and Goss ('46) described a similar disease among cattle in California and correlated its incidence with forage high in molybdenum. Comar, Davis and Singer ('48) recently reported that analyses of forage grown in some Florida areas, long recognized as copper deficient, showed high molybdenum content. "Swayback" in lambs is another disease

of grazing animals which responded to copper therapy. The condition was first described by Stewart ('32) in England and was reported as a copper deficiency disease by Dunlop and Wells ('38). Analyses of forage from "swayback" areas by Innes and Shearer ('40) showed an adequate copper content, however, and the presence of a toxic element was suspected. The lead and zinc content of the forage was found to be above average (Shearer, Innes and McDougall, '40).

These findings suggest that the copper metabolism of the animal is affected by the presence in the diet of several different minerals — zinc, molybdenum, and possibly lead. A study of the interrelationships of these 4 minerals in the nutrition of the rat is reported in this paper.

EXPERIMENTAL PROCEDURE

The experimental work was divided into two parts — the first, a study of molybdenum toxicity in the rat, and the second, a study of the interrelationships of copper, molybdenum, zinc and lead in rat nutrition.

Experiment I — Molybdenum toxicity in the rat

Methods and results. At the time this work was planned, the studies by Neilands, Strong and Elvehjem ('48) and by Comar, Singer and Davis ('49) of molybdenum toxicity in the rat had not been reported. Therefore, the first experiment was designed to observe the effects on the rat of several levels of molybdenum added to two basal diets, viz., mineralized whole milk powder [1] and rat chow.[2] Whole milk powder was supplemented with copper, iron and manganese so that these minerals would not be limiting factors for growth and hemoglobin formation. Copper as $CuSO_4 \cdot 5H_2O$, iron as $FeSO_4 \cdot 7H_2O$, and manganese as $MnSO_4 \cdot H_2O$ were added to the whole milk powder so that it contained 6.3 μg of copper, 37.5 μg of iron, and 100 μg of manganese per gram. Analyses

[1] Klim Powdered Whole Milk, The Borden Company, New York, N. Y.
[2] Rockland Rat Chow, Arcady Farms Milling Company, Chicago, Ill.

of the pulverized rat chow showed it to contain 14.1 μg of copper, 406 μg of iron, and 88.6 μg of manganese per gram. Since copper therapy corrected molybdenum toxicity in grazing animals, it was thought that the higher copper content of rat chow might have a protective effect in molybdenum toxicity in the rat. Consequently, higher molybdenum levels were used with the rat chow diets. Molybdenum, as $Na_2MoO_4 \cdot 2H_2O$, was added at the following levels to the two basal diets: to mineralized milk powder — 0.00 (controls), 0.04, 0.08, and 0.12% Mo; to rat chow — 0.00 (controls), 0.04, 0.10, and 0.40% Mo.

Forty-eight weanling rats, of the Sprague-Dawley strain, were randomized (Fisher and Yates, '43) into 8 treatments with three males and three females on each treatment. The rats on the 8 treatments were then randomized into 6 battery blocks with one animal from each treatment in each block of cages. They were housed individually in wire-screen cages in an air-conditioned room. The mean initial weights of the 6 rats on each treatment ranged from 33 to 37 gm. Weekly weights were recorded for the 7-week experimental period and hemoglobin was determined colorimetrically as oxyhemoglobin (Sheard and Sanford, '29) at the end of the 4th, 5th and 6th weeks.

The mean weight change of the rats on the different diets for the 7-week experimental period is shown in figure 1.

Molybdenum had a toxic effect when fed to rats on either basal diet; the gain in weight was progressively less as the amount of molybdenum in the diet was increased. The hemoglobin levels of the rats on all treatments were well within the normal range; the values fell between 14.78 and 15.91 gm of hemoglobin per 100 ml of blood, with a mean of 15.50 and standard deviation of $\pm$ 0.90. The appearance of the animals on both basal diets and both diets with 0.04% Mo was normal. The coats of rats on those diets containing more molybdenum were often rough and the hair was fine and short like that of young rats.

To learn if copper would have a curative effect for the molybdenum-toxic rats, the three surviving groups showing pronounced symptoms of toxicity (rat chow + 0.10% Mo, milk powder + 0.12% Mo, and milk powder + 0.08% Mo) were divided into thirds. Two animals from each diet-group were

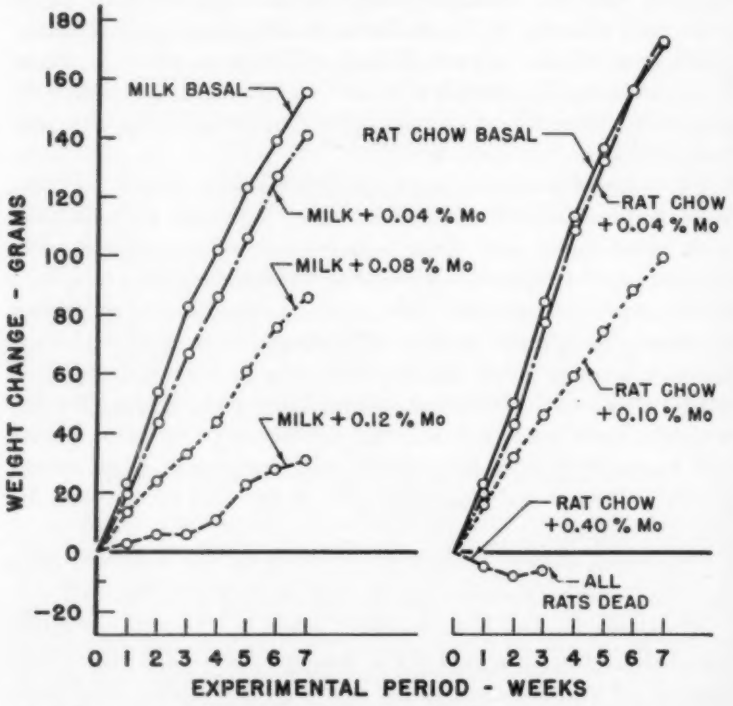

Fig. 1 Growth of rats fed two different basal diets with added molybdenum.

continued on the diet on which they had been for the past 7 weeks. Of the remaining 4 animals in each group, each of two received 0.20 mg and each of two received 0.40 mg of additional copper per day. The growth responses of the rats to these copper additions for a 5-week period are shown in figure 2. No effect was observed for the animals receiving

rat chow + 0.10% Mo or milk powder + 0.12% Mo. However, those animals fed 0.08% Mo responded to both 0.20 mg and 0.40 mg of additional copper daily. The mean gain in weight of those animals receiving 0.40 mg of copper was twice that of the controls.

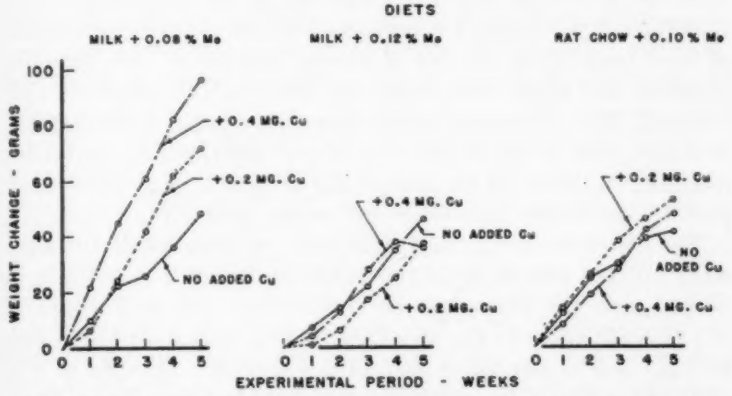

Fig. 2 Effect of added copper on the growth of rats fed molybdenum-toxic diets.

Experiment II — The interrelationships of copper, molybdenum, zinc and lead in the nutrition of the rat

Methods. Based on the results of experiment I, a factorial experiment was designed to study the interrelationships of copper, molybdenum, zinc and lead. Mineralized whole milk powder, supplemented as in experiment I, was used as the basal diet. The mineral levels selected for study were 0.01% Cu, 0.08% Mo, 0.50% Zn and 0.005% Pb. It had been demonstrated in experiment I that the addition of 0.08% Mo to this same basal diet resulted in moderate toxicity. A level of 0.01% Cu was thought adequate for the correction of this toxicity, since rats on this same level of molybdenum had responded at 10 weeks of age to a daily intake of copper which was approximately one-fourth that furnished by a

0.01% Cu level (based on an average food consumption of 10 gm per day). Past work in this laboratory (Smith and Larson, '46) had shown that a "zinc anemia" occurred in rats fed a diet of calf meal + 0.70% Zn. In recognition of the possibility of additive effects of the potentially toxic minerals, a level of 0.50% Zn was chosen for this study. The selection of a 0.005% Pb level was based on several studies of lead toxicity in the rat (Lederer and Bing, '40; Shields, Mitchell and Ruth, '39; Laug and Morris, '38; Horwitt and Cowgill, '37). The toxic levels reported ranged from 0.00035 to 0.01% Pb. Copper was added as $CuSO_4 \cdot 5H_2O$, molybdenum as $Na_2MoO_4 \cdot 2H_2O$, zinc as $ZnCO_3$ and lead as basic lead acetate (basic dry powder — for sugar analysis).

The above levels of the 4 minerals and zero levels for each were utilized according to the factorial design $2 \times 2 \times 2 \times 2$, which gave the following 16 treatments: (1) 0.00 (basal); (2) Mo; (3) Cu; (4) Zn; (5) Pb; (6) Cu + Mo; (7) Mo + Zn; (8) Mo + Pb; (9) Cu + Zn; (10) Cu + Pb; (11) Zn + Pb; (12) Mo + Cu + Zn; (13) Mo + Cu + Pb; (14) Mo + Zn + Pb; (15) Cu + Zn + Pb; (16) Mo + Cu + Zn + Pb. These diets were fed ad libitum for the duration of the experiment. In addition, vitamins A and D were supplied by administering a drop of percomorph oil [3] twice a week.

Eighty weanling rats (three males and two females on each of 16 treatments) were placed on experiment, as in experiment I. The mean initial weights of the 5 rats on each treatment ranged from 48 to 55 gm. Weekly weights were recorded and hemoglobin determinations were made at the end of the 7th and 8th weeks. Leukocyte counts were made at the end of the 8-week experimental period.

Results and discussion. The relative effects of the different treatments on growth are presented in figure 3. Good growth was obtained for the rats on all diets containing no added molybdenum. Although molybdenum retarded growth appreciably, the growth of those rats fed both zinc and molybdenum was considerably less than the growth of those fed only

[3] A–D Percomorph Oil, Abbott Laboratories, North Chicago, Ill.

molybdenum. An analysis of variance of these growth data showed that the main effects of molybdenum and zinc were each highly significant ($P = 0.01$), while the Mo $\times$ Zn interaction was significant ($P = 0.05$). A study of the growth trends in figure 3 indicated that a further breakdown of the data would be desirable to learn if zinc retarded growth significantly without molybdenum in the diet. When analyses of variance were made of the growth data of the 40 rats receiving molybdenum and of the 40 rats receiving no molybdenum, to

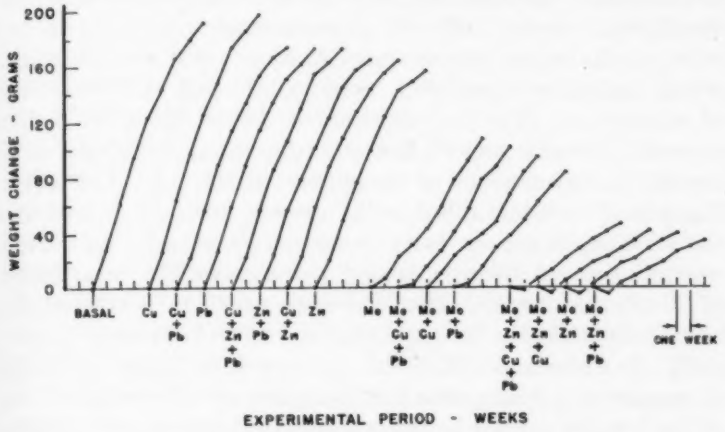

Fig. 3 Effect of treatment on rat growth.

test the significance of zinc in each instance, it was found that zinc did not significantly retard growth unless molybdenum was also present in the diet. Thus, a level of zinc which by itself did not retard growth, when present in the diet with molybdenum, caused a marked retardation beyond that caused by molybdenum alone.

While the growth of rats was markedly retarded by additions of molybdenum to the diet in both experiments, the results of experiment I indicated that the character of the basal diet influenced the severity of the molybdenum effect. Although the growth retardation was proportional to the

amount of molybdenum added to either basal diet, the relative effects differed somewhat with the two diets. The addition of 0.10% Mo to rat chow produced no more of a growth retardation than the addition of 0.08% Mo to mineralized milk powder. This difference may possibly be explained by the relative amounts of copper in the two diets; rat chow contained 14.1 μg of copper per gram, while mineralized milk powder had 6.3 μg per gram. However, with the copper-molybdenum relationship in mind, a comparison of the relative effects of identical molybdenum additions to two different basal diets under different experimental conditions is of interest. Neilands, Strong and Elvehjem ('48) at Wisconsin, using a purified basal diet, supplied the rat with 0.773 mg of copper per day; the addition of 0.04% Mo to this diet caused a weight retardation of approximately 40 gm in 6 weeks. In experiment I of the present study, a level of 0.04% Mo added to mineralized milk powder, which supplied the rat 0.063 mg of copper daily (based on an average daily food consumption of 10 gm), caused a mean weight retardation of 10 gm in 6 weeks. The Wisconsin workers corrected the effect of 0.04% Mo by supplying the rat 0.973 mg of copper daily. In experiment II of this study a daily intake of 1.0 mg of copper was inadequate for the growing rat for correction of the toxicity caused by the addition of 0.08% Mo to the diet. It will be remembered that when the daily copper intake of 10-week-old rats, fed 0.08% Mo, was changed from 0.063 mg to 0.263 mg, a growth response was observed. It would seem that both the character of the basal diet and the stress of rapid growth affect the molybdenum-copper relationship for the animal. It is believed that under the conditions of this experiment, a 0.01% Cu level in the diet was insufficient to counteract the toxicity of the 0.08% Mo level for the rapidly growing rat, which probably has a higher copper requirement than the more mature animal. Comar, Singer and Davis ('49) observed that molybdenum toxicity was much less pronounced in the mature than in the young rat. These workers, using a basal diet of whole milk powder diluted so

that approximately 50% of the diet was sucrose, produced a toxicity in rats with an 0.008% Mo level. This toxicity was corrected with 35 μg of copper per gram of diet. This copper level is not converted to daily copper intake for the rat as it seems probable that the food consumption was considerably less than the 10 gm per day average. Poor rat growth was obtained on their basal diet, and it would be predicted that a lower level of molybdenum would produce toxicity as a result of the poor nutritional status of the animal. The molybdenum-copper relationship was demonstrated, however.

The fact that zinc alone in the diet did not significantly retard growth is of some interest in view of the work of Smith and Larson ('46). These workers, feeding zinc to rats on a calf meal basal diet, found that both anemia and retarded growth occurred. Copper, which corrected the "zinc anemia," did not correct the poor growth, while liver extract corrected the poor growth but not the anemia. It appeared that zinc affected the metabolism of the rat in at least two ways — growth and hemoglobin formation — and that two different factors were active in correcting these effects. In the present experiment, either the level of zinc was too low to cause retarded growth or the difference was to be found in the basal diet; perhaps whole milk powder contained the factor(s) present in liver extract.

Hemoglobin levels, which were determined at the end of the 7th and 8th weeks of experiment II, are presented in table 1. An analysis of variance of these data showed that the effects of zinc in producing an anemia and of copper in correcting it were both highly significant ($P = 0.01$). The Cu × Zn interaction was of equal statistical importance. The Mo × Cu × Zn interaction was significant at the 5% level. This interaction cannot be interpreted as a result of this study alone. There were indications that copper was somewhat less effective in correcting the "zinc anemia" if molybdenum was also present in the diet. While anemia was observed in cattle on high-molybdenum forage in California, it was not related to molybdenum feeding in the present study.

Although the Zn × Pb interaction was not found to be statistically significant, the Zn + Pb treatment resulted in the lowest mean hemoglobin of any treatment. No conclusions can be drawn from this observation without further study.

An analysis of variance of the leukocyte count data showed no significant differences due to any treatment. The range of treatment means (in thousands per cubic millimeter) was 4.5 ± 0.76 [4] to 11.6 ± 3.38.

TABLE 1

Hemoglobin levels of rats on the various treatments

TREATMENT	MEAN HEMOGLOBIN [1] AT 7 WKS.	MEAN HEMOGLOBIN [1] AT 8 WKS.
	gm/100 ml	*gm/100 ml*
1. None	15.24	15.58
2. Mo [2]	14.18	14.14
3. Cu	15.06	15.66
4. Zn	10.45	11.06
5. Pb	15.32	15.27
6. Cu + Mo	14.51	14.88
7. Mo + Zn	12.61	11.55
8. Mo + Pb	13.76	13.66
9. Cu + Zn	14.20	15.43
10. Cu + Pb	14.48	15.65
11. Zn + Pb	7.57	8.96
12. Mo + Cu + Zn	13.95	13.70
13. Mo + Cu + Pb	14.36	14.60
14. Mo + Zn + Pb	11.43	11.14
15. Cu + Zn + Pb	13.55	14.54
16. Mo + Cu + Zn + Pb	13.65	13.72

[1] Mean hemoglobin of 5 rats (three males and two females).

[2] The various minerals were fed at the same levels throughout the experiment whether fed alone or in combinations; 0.08% Mo, 0.01% Cu, 0.50% Zn and 0.005% Pb.

SUMMARY

A toxicity was produced in rats by additions of molybdenum to two different basal diets, viz., mineralized whole milk powder and rat chow. Using the milk powder diet, a study was made of the interrelationships of copper, molybdenum,

[4] Standard error.

zinc and lead in the nutrition of the rat. The effects of molybdenum in retarding growth and of zinc in producing an anemia were confirmed. The ability of copper to correct this effect of molybdenum on growth was not clearly defined at the mineral levels used; however, copper corrected the anemia caused by zinc, as it had in past work. Zinc did not retard the growth of rats fed these milk powder diets, but the presence of both molybdenum and zinc in any diet resulted in significantly poorer growth than was caused by the addition of only molybdenum. Lead alone had no effect on growth or hemoglobin. At the levels fed, none of the minerals affected leukocyte count.

The demonstration of such relationships as these, viz., between copper and zinc, and molybdenum and zinc, and probably molybdenum and copper, emphasize the necessity for a consideration of the levels of all minerals in the diet before determining the requirements for any one. This type of study reaffirms the observation that a deficiency disease may not reflect merely a low level of a dietary essential, but an excess of one or more other nutrients which interfere with the normal metabolism of that essential dietary constituent.

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COMPARATIVE STUDIES IN NIACIN METABOLISM. THE FATE OF NIACIN IN MAN, RAT, DOG, PIG, RABBIT, GUINEA PIG, GOAT, SHEEP AND CALF [1]

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In 1946 the salient facts concerning the fate of niacin administered in large doses to various animal species were as follows: In man, rat and dog the chief metabolite excreted in the urine was N^1-methylnicotinamide (Huff and Perlzweig, '42, '43; Huff, '45; Sarett, Huff and Perlzweig, '42; Sarett, '42b). But only in the dog was complete saturation (close to 100% excretion) observed in terms of this metabolite, plus a small amount of unchanged niacin (Sarett, Huff and Perlzweig, '42; Sarett, '42b). In man and the rat, only 30 to 40% of the dose could be recovered as N^1-methylnicotinamide in the urine, even after prolonged feeding of niacin (Huff and Perlzweig, '42, '43; Huff, '45). As observed in the present study, the pig excreted not more than 10% of ingested niacin as N^1-methylnicotinamide. In the two common laboratory species of herbivorous animals, the rabbit and guinea pig, it was observed that with the rather sensitive and

[1] A preliminary report of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, Detroit, 1949 (Fed. Proc., *8:* 236, 1949).

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specific method available to us it was not possible to establish the excretion of N^1-methylnicotinamide, even after saturation with large doses of niacin either orally or parenterally administered (Huff and Perlzweig, '46; Handler, '44). The same was true for the horse (Huff, Pearson and Perlzweig, '46).

It should be further pointed out that the amount of niacin excreted, either unchanged or in forms measurable by microbiological assay with *L. arabinosus* (which include, in addition to nicotinic acid, the amide, nicotinuric acid, pyridine nucleosides and coenzymes I and II), did not account for more than 10% of the ingested doses in man, rat, dog, or guinea pig (the rabbit and horse excrete large amounts of unchanged nicotinic acid). Thus, it appeared that a large portion of ingested niacin was metabolized to other products than those which could be determined. Furthermore, it was observed that N^1-methylnicotinamide itself, when administered to man, rat, rabbit, and horse, was largely "destroyed" and could not be recovered in the urine (Perlzweig and Huff, '45; Perlzweig, Huff and Rosen, '46; Huff and Perlzweig, '46).

Knox ('46) demonstrated the presence in rabbit liver of an enzyme capable of oxidizing N^1-methylnicotinamide. Shortly thereafter, Knox and Grossman ('46) were able to isolate the oxidation product from the urine of a human subject ingesting large amounts of nicotinamide, and to identify it as the 6-pyridone of N^1-methylnicotinamide (hereafter referred to as the 6-pyridone). It became apparent that this oxidation product might account for the hitherto unaccounted for portion of the metabolized niacin in man, and possibly in the other species. However, the lack of a suitable quantitative method for estimating the amount of 6-pyridone in the urine delayed this study until the current year, by which time such a method was elaborated and found adequate for these purposes (Rosen, Perlzweig and Handler, '48; Rosen, Perlzweig and Leder, '49). With the aid of this method, niacin balance studies were carried out in man, rat, dog, pig, rabbit,

guinea pig, sheep, goat and calf. These also included studies on the fate of N^1-methylnicotinamide and of the 6-pyridone in these species. Some of the data on the herbivora are taken from our previously published studies (Huff, Pearson and Perlzweig, '46; Pearson, Perlzweig and Rosen, '49).

In this paper comparative data will be presented on the metabolism of niacin in man and 8 other mammalian species. It will be shown that the 6-pyridone is a major end-product of niacin metabolism in man, but not in the other species.

METHODS

The analytical methods employed were: niacin and related compounds active for *L. arabinosus* were determined by a microbiological procedure (Krehl, Strong and Elvehjem, '43); N^1-methylnicotinamide by the fluorometric procedure of Huff and Perlzweig ('47); and the 6-pyridone of N^1-methylnicotinamide by the method of Rosen, Perlzweig and Leder ('49).

EXPERIMENTAL

Ten, healthy young human subjects were employed. Urine samples (24-hour) were collected during a control period during which the subjects ate freely of diets of their own choice. The urines so collected, and all other urines in this study, were preserved with 3 ml of 50% acetic acid and toluene and were kept in a refrigerator. Subsequently two of the subjects were given 500 mg of nicotinamide daily for 5 days. After a lapse of one week, they were each given 224 mg of N^1-methylnicotinamide daily for three days. One week later, they received a single dose of the pyridone.

Two adult, female dogs were housed in individual wire metabolism cages and fed 20 gm per kilogram per day of a commercial chow.[5] Nicotinamide and its derivatives were given in single oral doses.

Five male rats, whose initial weights varied from 175 to 213 gm, were fed a complete "synthetic" ration. They were housed in individual metabolism cages and given single oral

[5] Purina Checkers.

doses of the experimental materials as desired. Two young pigs were used. They were fed stock rations and control urines were collected for two days. On each of the next three days, 2 gm of nicotinamide were given orally in gelatin capsules. After a 5-day interval, the procedure was repeated with N^1-methylnicotinamide and 5 days later with the pyridone.

The two guinea pigs were fed a mixture of raw cabbage, oats and a commercial chow,[6] while the two rabbits were fed cabbage and carrots only. Nicotinamide was given in single oral doses on 4 consecutive days. After a lapse of one week, equivalent doses of N^1-methylnicotinamide were given for two days and urine collected for 6 days. Urine was collected for 48 hours after dosage with the pyridone.

Details of the experimental procedures used for sheep, goats and calves have been described in detail elsewhere (Pearson, Perlzweig and Rosen, '49).

RESULTS AND DISCUSSION

Man

Of the known derivatives of nicotinic acid, only nicotinuric acid (Sarett, '42a) and N^1-methylnicotinamide (Huff and Perlzweig, '43) were established as constituents of human urine prior to the isolation of the 6-pyridone by Knox and Grossman ('46). Acid-hydrolyzable derivatives of nicotinic acid, including nicotinuric acid, constitute approximately 10% (0.5 to 1.5 mg as niacin) of the measurable metabolites in normal urine. Trigonelline does not appear to be a normal end-product of nicotinic acid metabolism, and its presence in the urine can be traced to the coffee and leguminous food in the ordinary diet (Huff, '45). Huff found that the daily excretion of N^1-methylnicotinamide in normal adult subjects on an average diet ranged between 4 and 12 mg, with an average of 7 mg. Hochberg et al. ('45) reported a daily output of 3.3 to 9 mg of N^1-methylnicotinamide in 6 normal subjects whose diet contained 23 mg of nicotinic acid.

[6] Purina "Rabbit Chow."

The data describing the excretion of N^1-methylnicotinamide and its pyridone by 10 healthy humans on average diets is summarized in table 1.

The range of values for N^1-methylnicotinamide in this small group of subjects is of the same order of magnitude as that found by Huff ('45) in 67 adults. The daily excretion of 6-pyridone in this group ranged between 3.6 and 12 mg

TABLE 1

Twenty-four-hour excretion of N^1-methylnicotinamide and 6-pyridone by healthy individuals on a normal diet

SUBJECT	SEX AND WT.	NM [1]	6-PYRIDONE	RATIO: 6-PYRIDONE / NM	TOTAL
	kg	*mg*	*mg*		
J. C.	M,79	7.8	9.2	1.18	17.0(14.3)[2]
A. D.	M,61	5.9	5.9	1.0	11.8(10.0)
F. R.	M,63	6.1	10.0	1.6	16.1(13.4)
I. L.	M,74	10.2	12.0	1.18	22.2(18.8)
M. E.	M,86	6.4	11.8	1.84	18.2(15.1)
H. K.	M,66	6.8	10.9	1.6	17.7(14.8)
S. H.	F,59	3.0	3.6	1.2	6.6(5.44)
R. S.	F,56	5.0	10.8	2.18	15.8(13.2)
B. P.	F,52	6.6	3.8	0.52	10.4(8.9)
I. P.	F,55	5.3	6.1	1.53	13.4(11.2)
Average		6.3	8.6	1.37	14.9(12.5)

[1] N^1-methylnicotinamide.

[2] The values in parentheses represent an equivalent amount of nicotinamide. To this amount should be added approximately 1 mg of niacin excreted as derivatives determinable by *L. arabinosus* assay.

with an average of 8.6 mg. The ratio of 6-pyridone to the N^1-methylnicotinamide excreted in 8 of the normal subjects varied from 1.0 to 1.8; a value of 0.52 was obtained in one subject and 2.18 in another. When converted to an equivalent amount of nicotinic acid, the total average excretion of 6-pyridone and N^1-methylnicotinamide was 12.5 mg. The average American diet has been estimated by Cheldelin and Williams ('43) to contain 17 mg of nicotinic acid per 2,500

cal. Thus, the combined excretion of both derivatives, plus 0.5 to 1.5 mg of niacin and derivatives determined by *L. arabinosus* assay, can be assumed to account for a large part (80%) of the nicotinic acid consumed daily by the average American adult.

TABLE 2

The excretion of niacin and its derivatives in two adults following the ingestion of nicotinamide, N¹-methylnicotinamide and 6-pyridone. (Values are in milligrams per 24-hour urine)

URINE	N¹-METHYL-NICOTINAMIDE		6-PYRIDONE		NIACIN		TOTAL	
	F.R.	I.L.	F.R.	I.L.	F.R.	I.L.	F.R.	I.L.
			Control — no supplement					
1	6	9	10	12	0.9	1.1	17 (14)[1]	22 (19)
			Supplement — 500 mg nicotinamide					
2	130	185	221	280	7.1	5.3	358(282)	470(394)
3	161	165	376	279	7.5	3.7	544(451)	448(374)
4	214	278	278	271	5.6	4.5	498(418)	554(470)
5	262	245	246	258	6.1	4.7	514(436)	508(429)
6	263	191	280	228	7.4	4.4	550(465)	433(357)
			Supplement — 224 mg N¹-methylnicotinamide					
12	57	35	45	41			102	76
13	70	48	37	24			107	72
14	47	34	40	48			87	82
			Supplement — 25 mg 6-pyridone					
15			22	23			22	23

[1] Values in parentheses represent an equivalent amount of nicotinamide.

In a recent preliminary report, Dean and Holman ('49) have applied a method for the determination of 6-pyridone to urines from adults and new-born children. The details of the method were not given. The normal values for 24-hour specimens obtained from 4 adults ranged between 10.8 and 14.9 mg; 4 infants excreted from 1.0 to 2.1 mg during the first 72 hours of life. An average value of 1.4 was obtained for the ratio of 6-pyridone to the N¹-methylnicotinamide excreted by the adult subjects.

Huff ('45) and Sarett ('42a) have previously studied the pathway of nicotinic acid metabolism in the human by meas-

uring various metabolites in the urine after the ingestion of nicotinic acid and its derivatives. Huff found that only 30 to 40% of an ingested dose of nicotinamide could be recovered in the urine as N^1-methylnicotinamide and the acid-hydrolyzable derivatives. Approximately 75% of an oral dose of N^1-methylnicotinamide could not be accounted for in the urine. Since large amounts of 6-pyridone have been isolated from human urine after the ingestion of nicotinamide (Knox and Grossman, '46), it seemed probable that the excretion of this derivative would explain the fate of a large portion of the nicotinamide and N^1-methylnicotinamide unaccounted for in this species.

Table 2 shows the results of studies on the relationship of the 6-pyridone to the metabolic fate of administered nicotinamide and N^1-methylnicotinamide. Calculated as an equivalent amount of nicotinamide, the combined excretion of N^1-methylnicotinamide, 6-pyridone and nicotinic acid accounted for 82 and 89% of the total amount of nicotinamide ingested during the test period. After the ingestion of nicotinamide, values ranging between 1.0 and 2.3 were obtained for the 6-pyridone: N^1-methylnicotinamide ratio; this range is of the same order as that observed in normal urines.

The results obtained when N^1-methylnicotinamide was given orally were unexpected. Of the total dose, only 30 to 40% was recovered in urine as N^1-methylnicotinamide and 6-pyridone. The fate of 180 mg of N^1-methylnicotinamide given subcutaneously was tested in one subject (F. R.). In the following 72-hour urine, 58% of the dose was recovered as unchanged N^1-methylnicotinamide and 33% as 6-pyridone; a total of 91% of the dose. Previously, Perlzweig and Huff ('45) had shown that 67% of a dose of N^1-methylnicotinamide administered intravenously could be recovered as such in the urine; the 6-pyridone, then unknown, was not determined.

From 88 to 92% of a single dose of the 6-pyridone was recovered in the next 48-hour urine unchanged, which indicates that the further metabolism of this compound in the body is unlikely.

Dog

Sarett ('42b) presented a thorough study of the metabolic fate of nicotinic acid and its derivatives in the dog. When this species is saturated with niacin or its amide, from 80 to 100% of the total dose can be recovered in the urine as N^1-methylnicotinamide, niacin and nicotinuric acid. N^1-methylnicotinamide, measured as "trigonelline" by Sarett ('42b), represents 75 to 95% of the total excretion. Since almost all of the ingested vitamin can be accounted for in the urine, it may be presumed that the dog is unable to oxidize N^1-methylnicotinamide to form the 6-pyridone. In the experiments described herein, the metabolism of nicotinic acid in the dog was re-evaluated with respect to the fate and excretion of N^1-methylnicotinamide and the 6-pyridone.

On the basis of the data presented in table 3, the 6-pyridone does not seem to be a normal end-product of niacin metabolism in the dog. None of this metabolite was excreted following a dose of nicotinamide, and only very small amounts after the ingestion of N^1-methylnicotinamide. One of the 24-hour control urines contained 2 mg of substances reacting as 6-pyridone with the method of analysis used. The identity of these substances is not certain, however, in view of the limitations of the analytical method.

The results obtained when N^1-methylnicotinamide was given orally are difficult to interpret. Since the excretion of N^1-methylnicotinamide accounts for almost all of an ingested dose of nicotinic acid, an oral dose of this compound was expected to be excreted unchanged in the urine. Failure to absorb the compound when given orally seemed to be the most likely explanation for the low recovery shown in table 3. This was tested in both dogs by the subcutaneous administration of the same amount of N^1-methylnicotinamide as was given orally. Whereas dog B excreted, unchanged, as much as 85% of a subcutaneous dose, dog A excreted but 2%. Hydrolysis of N^1-methylnicotinamide to trigonelline might also explain the poor recovery of N^1-methylnicotinamide when given

TABLE 3

Metabolism of nicotinamide, N¹-methylnicotinamide and 6-pyridone in the dog, rat and pig

SPECIES	SUPPLEMENT FED		EXCRETED IN URINE [1]				
	Compound	Amount	N¹-methyl-nicotin-amide	6-Pyri-done	Nico-tinuric acid [2]	Total Acct'd. for	Total Unacct'd. for
		mg/kg					
Dog	Nicotinamide	10	80	0 [3]	8	88	12
	N¹-methylnicotinamide	11	20	2 [3]		22	78
	6-Pyridone	2.5–4.0		69		69	31
Rat	Nicotinamide	100–200	50	3	15	68	32
	N¹-methylnicotinamide	100	50	3		53	47
	6-Pyridone	30–50		80		80	20
Pig	Nicotinamide	75–80	7	10	9	26	74
	N¹-methylnicotinamide	10	15	20		35	65
			(3.27)	(31.9)		55	45
	6-Pyridone	1		55			

[1] Values given as per cent of total dose excreted in the urine in 24 hours.

[2] Nicotinuric acid and derivatives as measured by *L. arabinosus* assay after *1* N HCl hydrolysis of the urine.

[3] Significance questionable due to low concentration.

orally. Samples of urine after oral ingestion of N^1-methylnicotinamide were analyzed for trigonelline according to the general method for estimating N^1-methyl derivatives of nicotinic acid in urine (Sarett, '43). The values for total methyl derivatives agreed very well with those previously obtained for N^1-methylnicotinamide, indicating that N^1-methylnicotinamide is not converted to trigonelline by the dog. The large percentage (69%) of unchanged 6-pyridone recovered in the urine after an oral dose of this compound indicates that the dog has a limited capacity for the further metabolism of this substance.

The excretion of 6-pyridone by the rat

Prior to the present studies on the excretion of 6-pyridone by the rat, the metabolism of niacin in this species was thought (with but one minor exception) to be similar to that observed in man. In contrast to man, nicotinuric acid was found to be hydrolyzed by the rat and to yield extra N^1-methylnicotinamide in the urine (Huff and Perlzweig, '42).

It was considered likely that the excretion of the 6-pyridone by the rat, as by man, would comprise a large portion of the hitherto unaccounted-for fraction after the ingestion of nicotinamide or N^1-methylnicotinamide. However, only 3% of an oral dose of nicotinamide or N^1-methylnicotinamide was excreted as the 6-pyridone. The significance of this small but definite amount of 6-pyridone formed from nicotinamide or N^1-methylnicotinamide is not clear. When 5 mg of 6-pyridone were given orally to these rats, 80% was recovered in the urine within 24 hours. Similar results were obtained when another group of rats were given these compounds intraperitoneally, thus eliminating the factor of absorption. On the basis of these results, it is unlikely that the rat does oxidize any appreciable quantity of N^1-methylnicotinamide to form the 6-pyridone. However, it should be added that, in preliminary studies, Hunter and Perlzweig ('49) have shown that rat liver slices are capable of oxidizing

small amounts of N^1-methylnicotinamide to the 6-pyridone. The present data do show, however, that N^1-methylnicotinamide is further metabolized in this species via another pathway. In this connection, Roth et al. ('48) have recently reported that nicotinic acid, labeled with C^{14} in the carboxyl group, or one of its metabolites, is decarboxylated by the mouse and that approximately 15% of an ingested dose of nicotinic acid can be recovered as respiratory carbon dioxide.

Attempts to measure the amount of 6-pyridone contained in normal rat urine were unsuccessful. It can be estimated, however, that less than 100 μg are excreted in 24 hours by rats kept on a purified diet containing 15% protein.

Pig

Although the requirement of the pig for nicotinic acid has been observed by many workers (Braude, Kon and White, '46; Hughes, '43; Powick, Ellis, Madsen and Dade, '47), the fate of nicotinic acid and its derivatives in this species has not been studied. N^1-methylnicotinamide has been established as an end-product of nicotinic acid metabolism in this species by Luecke et al. ('48), who have measured this derivative in the urine of pigs subsisting on purified rations containing various levels of casein.

The data presented in table 3 show that N^1-methylnicotinamide and the 6-pyridone are excreted by the pig following a dose of nicotinamide. However, the combined excretion of both derivatives and niacin accounts for only 26% of the total dose. It is difficult to evaulate the figures obtained for the chief metabolites excreted after the ingestion of N^1-methylnicotinamide, since they vary so widely (see values in parentheses, table 3). Only 55% of an oral dose of 6-pyridone was recovered, indicating that this derivative is further metabolized by the pig.

On the unsupplemented stock ration an average of 6 mg of N^1-methylnicotinamide was excreted in the 24-hour urine. In the control urine of one pig, 7.7 mg of 6-pyridone were

found, but none of this derivative could be detected in the control sample of the other animal.

Herbivora

In general, the behavior of the horse (Pearson and Luecke, '46; Huff, Pearson and Perlzweig, '46), rabbit (Handler, '44), and guinea pig (Handler, '44) in regard to nicotinic acid metabolism is unlike that of man, dog, pig and rat. These herbivora differ from other species in that they fail to excrete appreciable amounts of N^1-methylnicotinamide after the ingestion of nicotinic acid or its amide. In common with man, rat, dog, and pig, however, these species were found to be capable of "destroying" N^1-methylnicotinamide. On the basis of these observations it could not be stated whether herbivora methylate nicotinic acid and its amide, and the methylated derivative is further metabolized so rapidly that only small amounts are excreted, or whether niacin is metabolized via some other pathway. Since the N^1-methylnicotinamide-oxidizing enzyme was originally observed in rabbit liver, it seemed quite likely that the 6-pyridone would be a normal metabolite of niacin metabolism in this species and perhaps in other herbivora. To determine whether the 6-pyridone is an important end-product of niacin metabolism in herbivora, the fate of nicotinic acid and its derivatives was studied in 5 species of herbivorous animals; the rabbit and guinea pig, and the ruminants goat, sheep and calf.

The data in table 4 show that there is a small but significant increase in the excretion of 6-pyridone following the administration of nicotinamide to the rabbit and guinea pig. Only traces of a substance whose identity with the 6-pyridone is uncertain were found in the urine of the other herbivora (sheep, goat and calf) after the ingestion of nicotinamide. With the exception of the rabbit, the other species were capable of "destroying" from 84 to 96% of the orally administered N^1-methylnicotinamide. After the oral ingestion of N^1-methylnicotinamide, the rabbit continued to excrete significant

TABLE 4

Metabolism of nicotinamide, N¹-methylnicotinamide and 6-pyridone in the rabbit, guinea pig, goat, sheep and calf

SPECIES	SUPPLEMENT FED		EXCRETED IN URINE [1]				
	Compound	Amount	N¹-methylnicotinamide	6-Pyridone	Nicotinuric acid [2]	Total Acct'd. for	Total Unacct'd. for
		mg/kg					
Rabbit	Nicotinamide	90–100	0.5 [3]	3	43	46.5	53.5
	N¹-methylnicotinamide	100	17	25		42	58
	6-Pyridone	11–15		90		90	10
Guinea pig	Nicotinamide	150–200	0.5 [3]	5	8	13.5	86.5
	N¹-methylnicotinamide	160–200	0.5 [3]	4		4.5	95.5
	6-Pyridone	40–50		40		40	60
Goat	Nicotinamide	70–100	0.5 [3]	0.5 [3]	14	15	85
	N¹-methylnicotinamide	10	12	4		16	84
	6-Pyridone	1		35		35	65
Sheep	Nicotinamide	30–60	0.5 [3]	0.3 [3]	30	31	69
	N¹-methylnicotinamide	10	1 [3]	3		4	96
	6-Pyridone	1		11		11	89
Calf	Nicotinamide	30–70	0.6 [3]	0 [3]	35	36	64
	N¹-methylnicotinamide	10	2 [3]	3		5	95
	6-Pyridone	1		58		58	42

[1] Values given as per cent of total dose excreted in the urine in 24 hours.
[2] Nicotinuric acid and derivatives as measured by *L. arabinosus* assay after 1 N HCl hydrolysis of the urine.
[3] Significance questionable due to low concentration.

amounts of this derivative and the 6-pyridone during the next 5 days. This observation, and the fact that N^1-methylnicotinamide is excreted in relatively small amounts after the ingestion of nicotinic acid, would seem to imply that N^1-methylnicotinamide is not a normal metabolite of niacin metabolism in the rabbit. That large amounts of nicotinamide are not methylated by the rabbit and the guinea pig can be inferred from the studies of Handler ('44), who showed that these two species are not depleted of "labile methyl groups" when large amounts of nicotinamide (2%) are included in the diet for three weeks.

Additional evidence that N^1-methylnicotinamide is not a normal metabolite in this species was obtained when almost all of an oral dose of 6-pyridone was recovered as such in the urine. In the other herbivora studied, only 11 to 58% of the ingested dose of 6-pyridone was accounted for in the urine. Therefore, with respect to the guinea pig, sheep, calf, and goat the possibility remains that part of the ingested nicotinamide is metabolized via N^1-methylnicotinamide and 6-pyridone, and that the latter compound is further metabolized.

It was not possible to detect any appreciable quantity of 6-pyridone in the control urines of the calf, goat, and sheep. Both the guinea pig and rabbit excreted approximately 0.3 mg of this derivative in a normal 24-hour urine. The average 24-hour control excretion of N^1-methylnicotinamide in the various herbivora were: sheep, 2.8 mg; goat, 2.5 mg; calf, 4.1 mg; guinea pig, 0.1 mg; and rabbit, 1.1 mg.

SUMMARY

Metabolism studies were conducted in 9 mammalian species (man, dog, rat, pig, calf, sheep, goat, guinea pig and rabbit) on normal diets and after the ingestion of nicotinamide, N^1-methylnicotinamide and the 6-pyridone of the latter.

In a group of 10 healthy human subjects on a normal diet, from 3.6 to 12.0 mg of the 6-pyridone were excreted in 24 hours, with an average of 8.6 mg. The ingestion of large amounts of nicotinamide by two male subjects for a period

of 5 days resulted in the excretion of 35 to 45% of the dose as 6-pyridone and 30 to 40% as N^1-methylnicotinamide; thus, 82 to 89% of ingested nicotinamide could be accounted for in the urine as niacin, N^1-methylnicotinamide, and the 6-pyridone. Only 30 to 40% of an oral dose of N^1-methylnicotinamide was recovered in the urine of these two subjects as 6-pyridone and N^1-methylnicotinamide. Parenteral administration of N^1-methylnicotinamide led to a recovery of 91% in the urine as N^1-methylnicotinamide plus the 6-pyridone. Ingested doses of the 6-pyridone were almost completely excreted unchanged.

In the rat, only 3 to 5% of an oral dose of nicotinamide or of N^1-methylnicotinamide is excreted as the 6-pyridone. This animal excretes unchanged 80 to 90% of a dose of the 6-pyridone. N^1-methylnicotinamide accounts for 40 to 50% of an oral or intraperitoneal dose of N^1-methylnicotinamide or nicotinamide in this species.

In the dog, in which N^1-methylnicotinamide accounts for almost all of the ingested niacin at saturation, no 6-pyridone could be detected in the urine after the administration of nicotinamide and only small amounts after doses of N^1-methylnicotinamide. The 6-pyridone, when given orally to dogs, was largely recovered in the urine.

In the urine of the pig, N^1-methylnicotinamide accounted for 7% and 6-pyridone for 10% of an oral dose of nicotinamide. Almost half of the dose of 6-pyridone administered to the pig was not recovered in the urine.

The rabbit excreted less than 4% of a total dose of nicotinamide as N^1-methylnicotinamide and the 6-pyridone. However, in response to an oral dose of N^1-methylnicotinamide, 17% appeared in the urine as N^1-methylnicotinamide and 25% as 6-pyridone. This indicates that N^1-methylnicotinamide is not a normal metabolite of niacin in this species. No appreciable amounts of 6-pyridone are "destroyed" by the rabbit when given orally.

Relatively small amounts of 6-pyridone and N^1-methylnicotinamide are found in the urine of the guinea pig, sheep, calf, and goat, normally and after large doses of nicotinamide

and N^1-methylnicotinamide. From 42 to 89% of an oral dose of the 6-pyridone could not be recovered in the urine of these species. Only 4 to 16% of an oral dose of N^1-methylnicotinamide could be accounted for in the urine of these herbivora.

Apparently, in man, dog, rat and pig (and possibly other omnivora and carnivora?) excesses of niacin are largely disposed of by methylating processes. On the other hand, in the herbivora (rabbit, guinea pig and the polygastric species) niacin is probably disposed of by pathways other than those involving methylation.

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PHYSIOLOGICAL PROPERTIES OF SODIUM CARBOXYMETHYL STARCH [1]

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TWO FIGURES

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When a mixture of starch and alkali is treated with chloracetic acid, or sodium chloracetate, a water-soluble starch ether known as carboxymethyl starch is obtained. If one uses the simplified formula $(-O-Gl(OH)_3-O-)$ to represent the anhydroglucose repeating unit present in starch, then $(-O-Gl(OH)_2(OCH_2-COOH)-O)$ would represent a mono-substituted carboxymethyl ether of this repeating unit. Such a starch derivative is described as having a degree of substitution of 1.0. Fully etherified starch would have a degree of substitution of 3.0.

The modification of the starch molecule may bring about a change in its physiological action. It has been reported by some observers (Brown and Houghton, '41; Werle, '41; Rowe et al., '44; Shelanski and Clark, '48) that a modified cellulose preparation, sodium carboxymethylcellulose, is harmless physiologically. These workers believe that there is no evidence to indicate that sodium carboxymethylcellulose should not be incorporated into foods or pharmaceutical preparations designed for human or animal consumption. However, Rowe et al. ('44) observed incidentally that the intestinal contents of animals fed sodium carboxymethylcellulose were bulky and the feces soft. The laxative effect

[1] Aided by a grant from the Corn Products Refining Company.

apparently went unnoticed until recently, when it was put to clinical use (Fittipoldi and Davis, '48).

The present study has been carried out to determine the physiological properties of sodium carboxymethyl starch. Two modified starches were studied. One of them, called starch R 9, has a high degree of substitution (1.0) and contains 4.7 to 5.8% of sodium on a dry basis. It dissolves in hot or cold water to yield colloidal solutions having a high viscosity. The other sodium carboxymethyl starch is called starch R 10. Chemically speaking, it is identical with R 9 except that the degree of substitution is much lower (from 0.01 to 0.05). Its sodium content ranges from 0.4 to 0.55%. As regards physical properties, R 10 is quite similar to ordinary corn starch. It is insoluble in cold water and is dispersed in water by heating or by the addition of alkalies.

EXPERIMENTAL

Feeding tests on rats

Groups of weanling rats, males and females separated, were fed the experimental diets. The basal diet in per cent by weight (except one group, diet D in table 1) consisted of casein 25, carbohydrate (corn starch, R 9, R 9 + corn starch, or R 10) 45, lard 14, yeast 8, mixed salts 5, cod liver oil 3. In addition, wheat germ oil was added to the diet twice a week. Groups of weanling rats fed the diet containing regular corn starch were used as controls. All animals were weighed weekly and the general condition was observed. Each diet was fed to a group of 7 to 12 rats of both sexes. The results are summarized in table 1 and in figures 1 and 2.

It will be seen from table 1 that the R 9, a starch of a high degree of carboxymethyl substitution, caused diarrhea in the rat, even when the concentration of R 9 in the diet was as low as 5%. Feeding a diet with a high percentage (45%) of R 9 led to the death of the animals within a week. On autopsy, most of the rats showed hemorrhage from the mucosa of the stomach and distention of the cecum with fecal fluids. Iodine

TABLE 1

The effects of the various proportions of modified starches on the general condition of rats

DIET	DIETARY MODIFICATIONS	GENERAL CONDITION
A	Regular corn starch, 45%	Good.
B	R 9, 45%	Marked diarrhea and diuresis observed on the day after beginning feeding. Survival time varied from 4 to 7 days. Weight lost abruptly.
C	R 9, 20%, plus corn starch, 25%	Moderate diarrhea observed on day after beginning feeding, but there seemed to be no sign of diuresis. When pressure was applied to the abdomen of these rats, fluid stools readily flowed out. Only one died during the experimental period. No sign of adaptation was observed. The degree of diarrhea was more severe in males than in females.
D	R 9, 20%; corn starch, 25%; lard, 20%; casein, 44%	Moderate diarrhea observed on the second day of feeding, but there seemed to be no sign of diuresis. Both males and females showed degree of diarrhea comparable to that of the corresponding group on diet C.
E	R 9, 10%, plus corn starch, 35%	A less severe diarrhea than that in groups on diet C and D was observed on the second day of feeding. Males showed more diarrhea than females. No adaptation was observed. Rats weighing more than 136 gm also showed the same degree of diarrhea.
F	R 9, 5%, plus corn starch, 40%	Slight diarrhea was observed on the third day of feeding. No adaptation was observed.
G	R 10, 45%	Slight diarrhea comparable to that of the corresponding groups of diet F. Complete adaptation occurred after three months of continued feeding.

tests of the contents of the cecum and the colon showed no sign of starch. Adaptation occurred only when the modified starch of lesser substitution (starch R 10, diet G, table 1) was used.

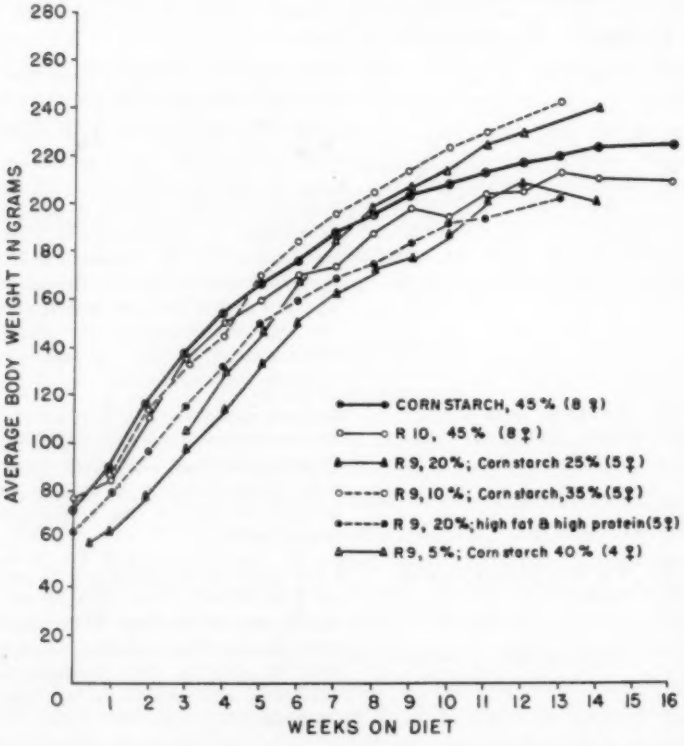
AVERAGE BODY WEIGHT IN GRAMS
WEEKS ON DIET
CORN STARCH, 45% (8 ♀)
R 10, 45% (8 ♀)
R 9, 20%; Corn starch 25% (5 ♀)
R 9, 10%; Corn starch, 35% (5 ♀)
R 9, 20%; high fat & high protein (5 ♀)
R 9, 5%; Corn starch 40% (4 ♀)

Fig. 1 Growth curves of female rats fed different dietary modifications. Corn starch, 45%, served as the control diet.

Feeding tests of carboxymethyl amylose and carboxymethyl amylopectin in rats. Since starch consists of two types of molecules, one a linear polymer of glucose called amylose and the other a branched polymer of glucose called amylopectin, each of these was fed in test diets to determine which

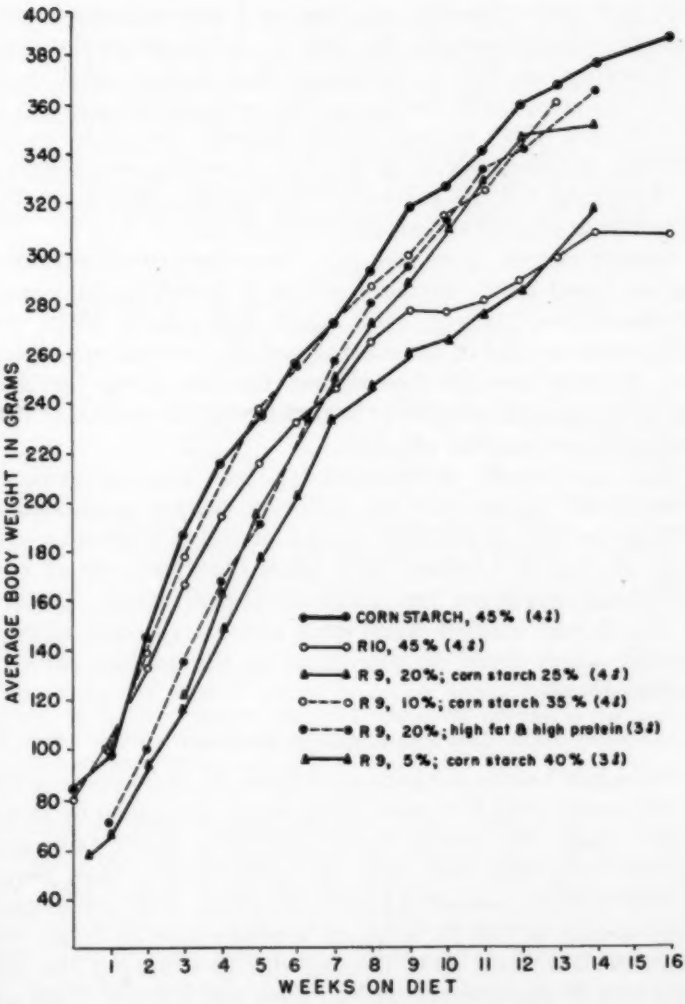

Fig. 2 Growth curves of male rats fed different dietary modifications. Corn starch, 45%, served as the control diet.

component is responsible for the diarrhea. The results showed that both carboxymethyl amylose and carboxymethyl amylopectin caused diarrhea. The degree of diarrhea in the group of weanling rats fed the carboxymethyl amylose (45% in the diet) was comparable to that in the corresponding group on diet B (R 9, 45%) in table 1, but diuresis seemed to be less marked. A lesser degree of diarrhea, however, was observed in the group fed the carboxymethyl amylopectin diet, 45%. This last group showed no sign of diuresis.

Growth curves. It can be seen from the growth curves of figures 1 and 2 that during a period of 4 months the growth of almost every group (R 9 up to 20%; R 10, 45%) was comparable to that of the controls fed the regular corn starch diet. Fertility records showed that ingestion of the test diets for 4 months did not affect the fertility of the animals. Normal lactation was not affected.

Histologic study. Pathologically, there were no gross lesions found in the rats fed different dietary modifications (R 9 up to 20%; R 10, 45%) over a period of 6 months. Sections of liver and kidney were taken from two rats of each group and prepared for histopathological study. In none of the tissues studied were there any microscopic changes present which could be attributed to the sodium carboxymethyl starches.

Ingestion tests on humans

Ten adult human subjects aged from 27 to 36 participated in this study. The R 9 cooked with water was taken by mouth twice a day. The intake was increased by 10 gm daily until diarrhea was observed. R 9, the starch with a high degree of substitution, caused diarrhea in adults with an average body weight of 148 lb. when an average dose of 60 gm was reached. The least dose causing diarrhea among the subjects was 40 gm, and the highest dose was 100 gm. Only one subject, weighing 225 lb., showed no effect whatever after taking 100 gm. Abdominal discomfort was found to be a general concomitant complaint.

Digestibility of the modified starches

Modification of the starch molecule may bring about a change in its digestibility. The relationship between the degree of substitution of modified starch and the change in its digestibility has also been studied.

Both the modified Wohlgemuth method (Koch, '37) and the modified Willstatter method (Schmidt et al., '34) were used in estimating the digestibility of both the boiled and unboiled starches throughout the study. The enzymes used in conducting the experiments were fresh saliva and pancreatin. The former was collected by chewing paraffin, usually one hour after breakfast. The saliva thus collected was filtered first through gauze and then coarse filter paper. In the incubation period of the unboiled starch solutions, frequent shaking was performed in order to keep the starch in suspension (except starch R 9, which can be dissolved in cold water).

TABLE 2

Digestion of starches by salivary amylase and pancreatin, using the modified Wohlgemuth method

STARCH	WOHLGEMUTH UNIT $D\ \frac{40°\ ^1}{30'}$			
	Salivary digestion		Pancreatin digestion	
	Boiled	Unboiled	Boiled	Unboiled
Soluble starch	83.25	3.58	22.50	0.70
R 9	53.25	62.50	22.50	15.63
R 10	2.58	2.08	2.50	0.54
Corn starch	1.25	1.79	0.63	0.39

[1] This means that at 40°C. 1 ml of the enzyme solution (undiluted saliva in the case of salivary digestion and 0.5% pancreatin solution in the case of pancreatic digestion) converts amounts in milliliters of 1% soluble starch solution into the achrodextrin stage in 30 minutes.

Results obtained with the modified Wohlgemuth method. Both the minimum concentration of enzyme at which the erythrodextrin reaction just persists and the minimum concentration at which the achromic point has been reached were recorded. The latter point was used to denote the digestibility of each starch. The average results of three tests on each starch are shown in table 2.

TABLE 3

Salivary and pancreatin digestion of the starches, using the modified Willstatter method[1]

STARCH	MALTOSE, IN MG			
	Salivary digestion		Pancreatin digestion	
	Boiled	Unboiled	Boiled	Unboiled
Soluble starch	67.69	33.37	102.66	36.22
R 9	18.56	8.16	22.55	11.15
R 10	69.63	15.01	94.88	10.59
Corn starch	26.30	15.87	76.85	13.08

[1] One milliliter of a 1 to 10 dilution of saliva was used in salivary digestion and 1 ml of a 0.1% pancreatin solution was used in pancreatin digestion. Fifteen milliliters of 2% starch solutions were used in all experiments.

Results obtained with the modified Willstatter method. The results using this method were expressed in milligrams of maltose as derived from the thiosulphate titration at the end of the hydroylsis of starch by amylase. The average data from three tests on each starch are shown in table 3.

It will be seen from table 2 that starch R 9, both boiled and unboiled, is easily digested down to the achrodextrin stage by the amylolytic enzymes of either saliva or pancreatin, resembling soluble starch in this respect; while starch R 10 is poorly dextrinized by both enzymes, resembling corn starch in this respect. On the other hand, as is shown in table 3, R 9 is very resistent to saccharification by both the pancreatin

and salivary amylase, while R 10 is as susceptible to saccharification as corn starch or soluble starch.

DISCUSSION

In an attempt to determine the physiological mechanism underlying this diarrheal effect, it was found that it is not due to the presence of free chloracetic acid, since sodium fusion of R 9 showed an absence of chloride. Nor is it due to high sodium content, since feeding a diet with a sodium acetate content comparable to the sodium content of R 9 in diet B (table 1) did not cause diarrhea or diuresis. Increasing the protein and fat content of the diet (diet D, table 1) did not afford any protection. A group of rats weighing more than 136 gm each exhibited the same degree of diarrhea as the weanling rats. Furthermore, the feeding of the alkali-treated starches which are used for the preparation of R 9 and R 10, and which differ from R 9 and R 10 only in that they have not been carried through the process of carboxymethylation, did not cause diarrhea or diuresis.

The study of the digestibility of the starches showed that both the boiled and unboiled R 9 are easily digested by both the salivary and pancreatic enzymes down to the achrodextrin stage, while R 10 is not so readily digested. In respect to dextrinization, R 9 resembles boiled soluble starch and R 10 resembles corn starch. However, the reducing sugar determinations (table 3) show that this reaction of R 9 does not go much beyond the achrodextrin stage. As was stated above, R 9, the highly substituted starch, is very resistant to saccharification by both pancreatic and salivary amylase, while R 10 is as susceptible to saccharification as corn starch or soluble starch. The digestibility of corn starch is not significantly altered by substitution with carboxymethyl groups to the extent to which the latter were present in R 10. However, further substitution, to the extent characteristic of R 9, results in enhanced susceptibility to dextrinization accompanied by resistance to saccharification to the maltose stage. It appears unlikely that the resistance to saccharification of

R 9 is responsible for the diarrhea-producing action, since R 10, which has a digestibility comparable to that of corn starch, also causes diarrhea when given in sufficient amounts.

All the evidence presented above would seem to indicate that the carboxymethyl group substituted in the starch molecule may be responsible for the observed effects. However, the problem of the physiological mechanism by which this diarrhea occurs has not been resolved.

SUMMARY

Carboxymethyl starch of a high degree of substitution (starch R 9) causes diarrhea when fed to rats in concentrations as low as 5% of the diet, and when fed to humans at an average level of 60 gm per day. High concentrations of R 9 in the diet of rats (45% of the diet by weight) cause death within one week. Lower concentrations do not impair growth, even though they cause diarrhea.

Carboxymethyl starch of a low degree of substitution (starch R 10) produces a milder diarrheal reaction than starch R 9 in rats receiving it in a concentration of 45% of the diet.

Carboxymethylamylose produces diarrhea more readily than does carboxymethylamylopectin.

Starch R 10 shows no important difference from unmodified corn starch in regard to dextrinization and saccharification by salivary and pancreatic amylase. Starch R 9 shows an increased susceptibility to dextrinization but a decreased susceptibility to saccharification.

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